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Apoptosis pathways in (pre)malignant gastrointestinal tumours : potentials for intervention

Jalving, Mathilde

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Apoptosis pathways in (pre)malignant gastrointestinal tumours: potentials for intervention

M. Jalving

**Apoptosis Pathways in (Pre)malignant
Gastrointestinal Tumours:**
Potentials for Intervention

Cofolon

The research presented in this thesis was financially supported by the Dutch Cancer Society: grants RUG 2000-2286 and RUG 2005-3361, Groningen Institute for Drug Exploration, Dutch Digestive Diseases Foundation: grant WS 2001-31, De Cock Stichting, Nijbakker-Morra Fund and the Faculty of Medicine, University Medical Center Groningen.

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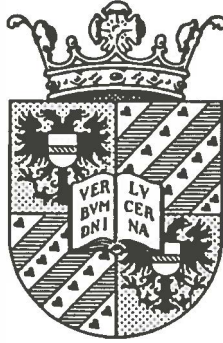
Stellingen

Behorende bij het proefschrift

“Apoptotic Pathways in (Pre)malignant Gastrointestinal Tumours: Potentials for Intervention”

1. De toekomst van darmkanker chemopreventie ligt in het toepassen van combinaties van weinig toxische geneesmiddelen die aangrijpen op specifieke signaaltransductieroutes (dit proefschrift).
2. Het feit dat recombinant humaan TRAIL apoptose kan induceren in hoog dysplastische colorectale adenomen geeft aan de TRAIL-apoptose route ook in premaligne tumoren functioneel kan zijn (dit proefschrift).
3. De opmerkelijke overexpressie van de pro-apoptotische TRAIL receptoren in colorectale adenomen en carcinomen wordt gedeeltelijk veroorzaakt door β -catenine (dit proefschrift).
4. Aspirine kan intrinsieke en verworven resistentie voor recombinant humaan TRAIL-geïnduceerde apoptose in colonkankercellijnen opheffen (dit proefschrift).
5. Het is niet nodig om patiënten die langdurig proton-pomp remmers gebruiken te controleren op het ontwikkelen van dysplastische fundic gland poliepen (dit proefschrift).
6. Een aspirine- en statine bevattende cocktail ter voorkoming van meerdere ziekten, waaronder hart-en vaat ziekten en kanker, is nog niet wenselijk (Journal Epidemiology and Community Health 2006;60:213-7).
7. In de ontwikkeling van anti-tumor middelen zijn nieuwe, originele criteria voor de evaluatie van tumorrespons noodzakelijk (Nature Reviews Cancer 2006;6:409-414).
8. De succesvolle ontwikkeling van gerichte therapie, zoals bevacizumab en erlotinib, onderstreept het belang van doorlopend fundamenteel en translationeel onderzoek.
9. De aanwezigheid van een moleculair target is geen garantie voor een respons op een op dat target gerichte therapie (Journal Clinical Oncology 2006;24:213-6).

10. Het feit dat de biologen-term "arts-achtige" synoniem staat voor "compleet gebrek aan moleculaire interesse en kennis" geeft aan dat vooroordelen een optimale samenwerking kunnen belemmeren.
11. Om zijn vooruitstrevendheid te behouden en effectiviteit te verhogen moet de Junior Scientific Masterclass verder en breder kijken dan het MDPHD programma lang is.
12. De cultuur van "je hoofd niet boven het maaiveld mogen uitsteken" staat haaks op de ambities van Nederland om een kenniseconomie te worden.
13. Een promotie is geslaagd als de uitspraak van W.B. Yeats "Education is not the filling of a pail, but the lighting of a fire" ook aan het eind nog waar is.
14. De wereld is een boek, wie niet reist...leest slechts een bladzijde (Augustinus, 400AD).



RIJKSUNIVERSITEIT GRONINGEN

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Potentials for Intervention**

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Chapter 1

Introduction

Colorectal cancer is a leading cause of morbidity and mortality in Western countries ¹. Tumours can arise anywhere in the colon, however, the majority of sporadic cancers are found distal to the splenic flexure. Malignant behaviour is characterised by autonomous and unrestrained growth, invasion into the surrounding tissue and metastasis, mainly to the regional lymph nodes, the liver and the lungs. Hereditary and environmental, including dietary, factors contribute to the development of colorectal neoplasia ². Most, if not all, colorectal carcinomas arise from pre-existing, premalignant tumours known as adenomas ¹. Normal intestinal epithelium progresses through adenoma to carcinoma due to an accumulation of (epi)genetic alterations, causing a gradual disturbance in the balance between cell division (proliferation) and programmed cell-death (apoptosis) ¹. Colorectal carcinogenesis is hallmarked by a relative resistance to apoptosis and, once the disease has progressed past the early stages, treatment options are limited due to intrinsic and acquired resistance to chemotherapeutic drugs. The relatively slow evolution of normal epithelium to colorectal carcinoma, however, does leave a large window of opportunity for prevention and therapy during the early stages of the disease.

Cancer chemoprevention is the administration of pharmacological agents to prevent cancer development. Epidemiological studies have repeatedly shown that non-steroidal anti-inflammatory drugs (NSAIDs), which are currently widely prescribed as painkillers and anti-inflammatory drugs, reduce the risk of colorectal cancer ³. This has been confirmed in both animal studies and in prospective studies in patients at increased risk for colorectal carcinoma. In vitro studies have started to unravel the mechanisms responsible for this effect which include the induction of cell-cycle arrest, inhibition of proliferation and induction of apoptosis ³. NSAIDs, however, do not completely prevent colorectal cancer and chronic use is associated with an increased risk of gastrointestinal ulceration and bleeding and disruption of renal hemodynamics ⁴. Newer NSAIDs, which are more specific and therefore have less gastrointestinal side-effects, have recently been shown to increase the risk of cardiovascular disease and are therefore less suitable in the preventive setting. Chemoprevention with NSAIDs is, at this moment, only feasible for a small subset of patients with an extremely high risk of colorectal cancer. In clinical practice, this means that only patients with the inherited cancer syndrome caused by a mutation in the adenomatous polyposis coli (APC) gene, known as familial adenomatous polyposis (FAP), benefit from chemoprevention with NSAIDs. One possible strategy to expand the indications for chemoprevention is the identification of drugs which can be combined with low dose NSAIDs to

increase the efficacy while keeping the side-effects at an acceptable level 5. The aim of the first part of this thesis is to investigate the potential of the cytokine tumour-necrosis-factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the prevention and treatment of colorectal cancer. **Chapter 2** discusses the rationale of combining NSAIDs with (novel) drugs, such as TRAIL, which target specific signalling pathways in malignant or premalignant cells to increase the effectivity of colorectal cancer chemoprevention.

Apoptosis results from the regulated activation of a pre-existing death programme that is encoded in the genome. This programme can be activated by genetic defects, cell damage due to radiation or toxic agents and also by ligand activation of cell-surface death receptors. TRAIL is a member of the TNF family which also includes TNF- α and Fas ligand, and induces apoptosis in a wide variety of cancer cell lines but not in normal tissue ⁶. TRAIL induces apoptosis by binding to its pro-apoptotic receptors DR4 and DR5 leading to the activation of proteins which can directly, or indirectly, initiate apoptosis. The pro-apoptotic TRAIL receptors are widely expressed in colorectal adenomas as well as in colorectal carcinomas, but not in normal colorectal epithelium ⁶⁻⁸. Colorectal carcinoma cell lines and human colon carcinoma xenografts have been shown to be sensitive to TRAIL-induced apoptosis and the immunohistochemical expression patterns of DR4 and DR5 suggest that adenomas could also be sensitive to TRAIL ^{6,9}. In **chapter 3** the investigation of the TRAIL sensitivity of human colorectal adenoma cell lines and human colorectal adenomas is described.

The increased expression of the pro-apoptotic TRAIL receptors in colorectal adenomas and carcinomas in comparison with normal colorectal epithelium renders these tumours potentially sensitive to TRAIL induced apoptosis. The reason for this increased expression is unknown. Knowledge regarding the regulation of receptor expression is important both for the understanding of the biological pathways involved as well as to predict clinical effect and potential interactions between TRAIL and other drugs. The Wingless-Int (Wnt) pathway is important in the initiation and progression of the majority of colorectal carcinomas. Mutational inactivation of the *APC* gene, often a first step in colorectal carcinogenesis, leads to disruption and consequent activation of the Wnt pathway, resulting in the transcription of genes which are involved in apoptosis and proliferation ¹⁰. Activation of the Wnt pathway increases during the transition from normal colorectal epithelium to adenoma to carcinoma ¹⁰. The fundamental role of the Wnt pathway in colorectal cancer in combination

with its increase in activity during the adenoma-carcinoma sequence makes this pathway an interesting starting point in the investigation of TRAIL-receptor regulation. In **chapter 4** the investigation of the role of the Wnt pathway in the expression levels of DR4 and DR5 is described.

Although recombinant human (rh)TRAIL is an interesting novel anti-cancer drug and phase I studies using rhTRAIL-based therapies are ongoing, a potential problem in the clinical application of rhTRAIL is intrinsic and acquired resistance to rhTRAIL-induced apoptosis¹¹. Not all colon cancer cell lines are sensitive to rhTRAIL-induced apoptosis and continuous incubation with rhTRAIL has been shown to lead to the development of a rhTRAIL resistant cell population^{11,12}. A number of studies suggest that rhTRAIL and NSAIDs may act synergistically in the induction of apoptosis. NSAIDs have been shown to influence the levels and localisation of proteins involved in TRAIL-induced apoptosis and the combination of NSAIDs and TRAIL *in vitro* has been shown to be more effective than either drug alone¹³. In **chapter 5** the *in vitro* modulation of rhTRAIL induced apoptosis by NSAIDs is investigated, along with possible mechanisms responsible for this effect.

For a thorough understanding of carcinogenesis it is necessary to understand the effect of mutations and changes associated with the progression from normal tissue to dysplastic tissue. Besides being at increased risk of colorectal cancer, patients with FAP are also at increased risk for the development of fundic gland polyps (FGPs)¹⁴. These polyps, which were traditionally thought to be benign, have occasionally been reported to become malignant in patients with FAP. FGPs can also occur in patients without a germline APC gene mutation. However, these non-FAP FGPs occur less frequently and in smaller numbers than FGPs in FAP patients. While both low- and high-grade dysplasia are frequently found in FAP-FGPs, the risk of dysplasia in FGPs from non-FAP patients is extremely low. The aims of the second part of the thesis are to study the aetiology and the malignant potential of FGPs.

Chapter 6 describes an unusual case of a patient, without a history suspect for FAP, who was found to have high-grade dysplasia in a FGP. The differences in risk of FGP development and in the risk of dysplasia in FGPs between FAP and non-FAP patients suggest that FGPs develop differently in these two patient groups. This is studied in **chapter 7** where FGPs from both FAP and non-FAP patients are examined. The investigation is concentrated around the role of aberrant Wnt pathway activation, which is thought to be involved in FGP development, and its association with dysplasia, apoptosis and proliferation.

The potential for dysplasia in FGPs has raised some questions regarding the use of proton pump inhibitors (PPIs). Patients using PPIs have been reported to have an increased risk of FGPs and some authors have therefore recommended endoscopic surveillance for patients on long-term PPI therapy. Whether the actual risk of FGPs is increased is still a topic of debate since the few available studies have shown contradictory results^{15,16}. How these FGPs develop during PPI therapy is unclear, as is the risk of dysplasia in these PPI-induced FGPs. **Chapter 8** describes a case-control study examining the risk of FGPs in patients with and without long term use of PPIs, the presence of dysplasia in these FGPs and also the aetiology of these polyps.

In **chapter 9** a summary of all results is given along with a general discussion regarding the interpretation, implications and potential applications of these results. This is followed by a discussion of the future perspectives and possible further research in this area.

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Chapter 2

Potential of combinational regimen with non-steroidal anti-inflammatory drugs (NSAIDs) in the chemoprevention of colorectal cancer

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Alimentary Pharmacology and Therapeutics 2005; 21: 321-339

Abstract

Background: Non-steroidal anti-inflammatory drugs (NSAIDs) are chemopreventive agents in colorectal cancer. NSAIDs do not, however, offer complete protection against adenoma and carcinoma development. There is increasing interest in combining NSAIDs with agents that target specific cell signalling pathways in malignant and premalignant cells.

Aim: To describe the current knowledge regarding the efficacy of peroxisome proliferator-activated receptor γ (PPAR γ) ligands, cholesterol synthesis inhibitors (statins), epidermal growth factor (EGF) signalling inhibitors and TNF-related apoptosis inducing ligand (TRAIL) against colorectal neoplasms and the rationale for combining these drugs with NSAIDs to improve efficacy in the chemoprevention of colorectal cancer.

Methods: A pubmed computer search of the English language literature was conducted to identify relevant papers published before July 2004.

Results: PPAR γ ligands and statins, both in clinical use, reduce the growth rate of human colon cancer cells *in vitro* and in rodent models. *In vitro*, preclinical *in vivo* and clinical studies have shown efficacy of (EGF) signalling inhibition in colorectal cancer. *In vitro*, TRAIL induces apoptosis in human colon cancer cells, but not in normal cells. These drugs have all been shown to interact with NSAIDs in colorectal cancer cells and/or in rodent models.

Conclusion: Combinational regimes are a promising strategy for the chemoprevention of colorectal cancer and should be further explored.

Introduction

Colorectal cancer is the second most common cause of cancer related death in Western countries ¹. An accumulation of (epi)genetic alterations causes normal intestinal epithelium to progress through adenoma to carcinoma ². Malignant behaviour is characterized by autonomous and unrestrained growth, invasion into the surrounding tissue and metastasis, mainly to the lymph nodes, the liver and the lungs. Hereditary and environmental, including dietary, factors contribute to the development of colorectal neoplasia. Hereditary non-polyposis colorectal cancer (HNPCC), caused by germline mutations in mismatch repair (MMR) genes, is responsible for approximately three percent of colorectal cancer cases ³. Familial adenomatous polyposis (FAP), caused by germline mutations in the *adenomatous polyposis coli* (APC) gene and *MYH*-associated polyposis, caused by bi-allelic germline mutations in the base-excision-repair gene *MYH*, each account for less than one percent of cases ³⁻⁵. In Western countries, the cumulative lifetime risk of developing colorectal cancer is approximately 5 % in the general population, but is higher in certain subgroups ⁶.

The high incidence, morbidity and mortality of colorectal cancer make effective prevention of this disease necessary ⁷. The relatively slow evolution of normal epithelium to adenomatous polyps to colorectal cancer results in a large window of opportunity for prevention and early therapy. Current prevention strategies concentrate on early detection by (bi)annual faecal occult blood testing in the general population and regular screening colonoscopy for individuals at increased risk ^{8,9}. Cancer chemoprevention is the administration of pharmacological agents to prevent cancer development ¹⁰. Goals of chemoprevention are to reduce cancer risk and improve quality of life and life expectancy as measured through declines in frequency of invasive surveillance procedures, surgical resections, incidence of neoplasia and cancer related deaths. Chemoprevention with micronutrients is interesting since calcium has been demonstrated to have a preventive effect on the development of colorectal adenomas, selenium was associated with a lower incidence of colorectal cancer and high dietary folate was found to be associated with a lower risk of colorectal adenomas ¹¹⁻¹³. Furthermore, aspirin and non-aspirin NSAIDs (non-steroidal anti-inflammatory drugs) and their derivatives have repeatedly been shown to reduce the risk of colorectal cancer ¹⁴. The effect of NSAIDs is, however, incomplete and is associated with an increased risk of gastrointestinal ulceration, bleeding and disruption of renal hemodynamics.

Two cost-effectiveness analyses strongly suggest that screening is more suitable than chemoprevention with aspirin for the general population^{15,16}. Strategies to improve the efficacy of chemoprevention include the identification of high-risk populations, the use of selective NSAIDs and the identification of combinations of drugs with a higher efficacy^{7,14,17,18}. This review discusses drugs that could, in the future, be combined with NSAIDs to increase efficacy of chemoprevention in colorectal cancer.

Chemoprevention with NSAIDs

NSAIDs are inhibitors of the cyclo-oxygenase (COX) enzymes which are involved in the production of prostaglandins. Epidemiological studies showed that aspirin or NSAID use leads to up to 50% risk reduction for colorectal cancer and that this risk reduction is related to the amount of aspirin used¹⁹. Observational studies in FAP patients showed regression of polyps during therapy with the non-selective NSAID sulindac¹⁹. Furthermore, NSAIDs were shown to inhibit chemically induced intestinal cancer in rodents⁷. Later studies showed that NSAIDs also suppress tumourigenesis in *Apc^{min/+}* mice¹⁴. *Apc^{min/+}* mice, used as a model for FAP, have a germline *APC* mutation in one allele and develop multiple polyps in the gastrointestinal tract. More recently, the different mechanisms of action of NSAIDs in colorectal carcinogenesis have been investigated.

NSAIDs reduce prostaglandin synthesis by inhibiting the activity of the COX enzymes, of which there are two major isoforms^{20,21}. COX-1 is expressed in many tissues and plays a central role in platelet aggregation and gastric cytoprotection while COX-2 expression is induced during inflammation and wound healing²². Interestingly, the COX-2 enzyme is overexpressed in 45-50% of colorectal adenomas and 80-85% of carcinomas²³. In *Apc^{min/+}* mice, deletion of the COX-2 gene resulted in reduced polyp formation as compared to COX-2-expressing *Apc^{min/+}* mice²⁴. Mechanisms through which COX-2 inhibition may exert its effects are increased apoptosis and inhibition of both proliferation and angiogenesis (Fig. 1)²⁵⁻²⁷. Apparently, COX-2 independent mechanisms are also involved since NSAIDs induce apoptosis in cells without COX-2 expression^{28,29}. One important mechanism appears to be the inhibition, by NSAIDs, of the interaction between the T-cell factor 4 (TCF-4) transcription factor and its co-activator β -catenin. Furthermore, NSAIDs influence the kinases which regulate activation of the transcription factor nuclear factor

kappa B (NF- κ B). Both TCF-4 and NF- κ B influence genes involved in cell growth, differentiation and apoptosis (Fig. 1)³⁰⁻³².

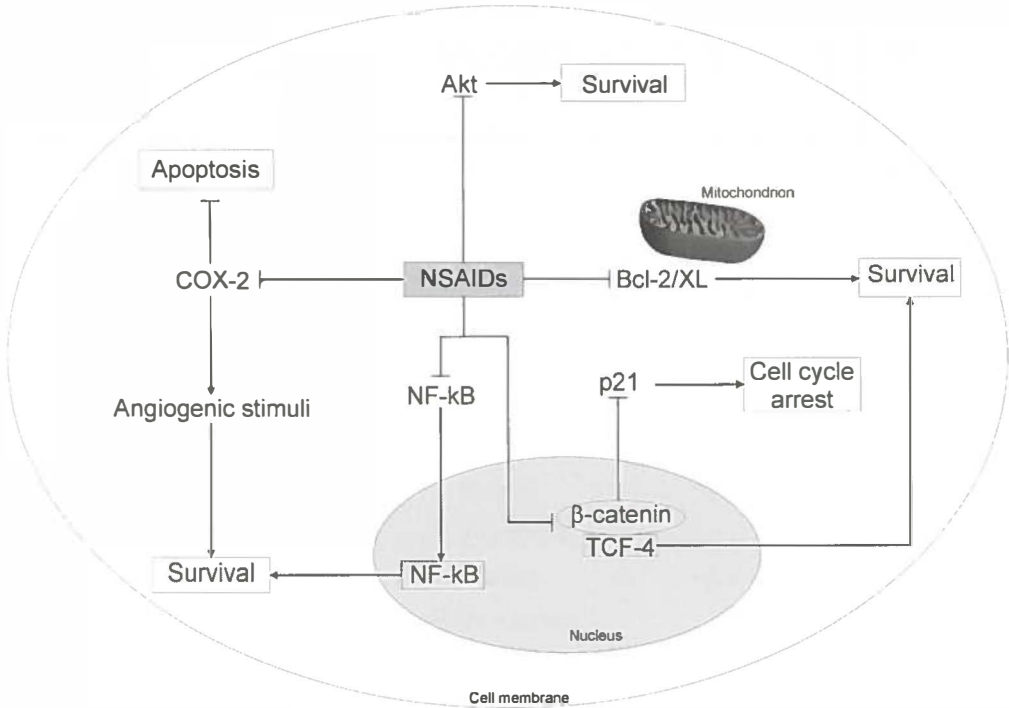


Figure 1: Molecular mechanisms that mediate the effects of NSAIDs in colon cancer cells. NSAIDs = non-steroidal anti-inflammatory drugs, NF κ B = nuclear factor kappa B, TCF = T-cell factor, COX = cyclo-oxygenase. Stimulates \longrightarrow . Inhibits ---| .

The cell-cycle inhibitory protein p21^{WAF1/Cip1}, regulated indirectly through TCF-4, is thought to be important in mediating the chemopreventive effects of NSAIDs³³. NSAIDs have also been shown to down-regulate several anti-apoptotic proteins including Bcl-2 family members^{34,35}. Finally, NSAIDs can induce apoptosis by blocking the activation of Akt, which is a key signalling component in cell survival³⁶. COX-independent effects, however, often occur at concentrations (for example 100-400 μ M indomethacin) that are much higher than those which can be reached clinically (maximum 10 μ M indomethacin).

In randomised clinical studies, the non-selective NSAID sulindac and the selective COX-2 inhibitor celecoxib have been shown to decrease the size and number of colorectal adenomas in FAP patients (Table 1).

Table 1: Effect of NSAIDs on adenoma incidence in randomised placebo controlled trials

Reference	Patients	N	Treatment (/day)	Reduction in polyp number	Reduction in polyp burden
Nugent ¹⁹¹	FAP	14	Placebo	-	None
			Sulindac 400 mg		71% of patients*
Ladenheim ¹⁹²	Sporadic adenoma	44	Placebo	19 %	-
			Sulindac 300 mg	39 %	
Steinbach ¹⁹³	FAP	77	Placebo	5 %	4.9 %
			Celecoxib 200 mg	12 %	14.6 %
			Celecoxib 800 mg	28 %*	30.7 %*
Giardiello ³⁸	FAP	22	Placebo	None	None
			Sulindac 300 mg	56 %*	65 %*
Labayle ³⁷	FAP	10	Placebo	22% of patients	-
			Sulindac 300 mg	100% of patients*	
Higuchi ¹⁹⁴	FAP	21	Placebo	None	None
			Rofecoxib 25 mg	6.8 %*	16.2 %*

NSAIDS = non-steroidal anti-inflammatory drugs, FAP = familial adenomatous polyposis, N number of patients, * significant difference compared to placebo.

However, in these studies in which duration of treatment varied between 4 and 9 months, few patients had complete resolution of polyps and polyp recurrence occurred within 3 months after discontinuation of treatment ^{37,38}. The reduction in polyp numbers in FAP patients persisted during long term treatment with sulindac (mean 63 months) ³⁹. However, five of twelve patients failed to complete this study due to disease progression, side-effects or poor compliance and one of the remaining seven patients developed rectal cancer. In a recent study in 41 young, phenotypically unaffected patients with a germ-line APC mutation, sulindac treatment failed to prevent the development of adenomas ⁴⁰. In populations at moderate risk for adenoma development high dose aspirin use (300-325 mg) was associated with a small reduction in the

incidence of adenomas (Table 2) ^{41,42}. In contrast, Baron *et al* found that 325 mg aspirin had no effect on adenoma development, while low dose aspirin (81 mg) was associated with a small reduction in adenoma occurrence ⁴³. It has been suggested that differences in trial design could explain these contrasting results ⁴³. In addition, several phase III chemoprevention trials with selective COX-2 inhibitors in populations at increased risk for colorectal cancer are currently in progress ⁴⁴.

In general these studies indicate that, although treatment with NSAIDs inhibits adenoma development and growth, this effect is far from complete and disease progression still occurs. The long-term use of NSAIDs is associated with a significant risk of complications. NSAID use increases the risk of gastrointestinal problems including ulcers, bleeding and perforation ⁴⁵. Inhibition of the synthesis of renal prostaglandin by NSAIDs may induce nephrotoxicity or adversely influence the renal function of patients treated with diuretics ⁴⁶. Gastrointestinal, but not renal, side effects can be reduced by the use of selective COX-2 inhibitors ^{47,48}. Alarming, the selective COX-2 inhibitor rofecoxib has recently been withdrawn from the market after preliminary results from a recent polyp trial showed an adverse cardiovascular side-effect profile (www.vioxx.com). It is unknown at this time whether this is a class-effect for the COX-2 inhibitor or a specific problem related to the specificity and relatively long half-life of rofecoxib ⁴⁹.

In summary, although a chemopreventive effect in colorectal cancer has been repeatedly demonstrated, NSAIDs alone are not suitable for chemoprevention of colorectal cancer in the majority of the population with an increased risk. In chemoprevention trials, a significant percentage of patients continued to develop colorectal adenomas despite NSAID use. In trials in FAP patients development of rectal cancer during NSAID use has been reported. This information, taken together with the above mentioned side-effects, has led to the conclusion that the risks of NSAID use outweigh the benefits in most people and that screening programs are a better option.

An increase in effectivity could tip the balance in favour of chemopreventive strategies for patients with an increased risk of colorectal cancer. One way of achieving this might be by combining NSAIDs with other drugs. These drugs should have chemopreventive effectivity, alone or in combination with NSAIDs, along with a low toxicity. Four classes of drugs which might, in future, meet these requirements, will be discussed in the following sections.

Table 2: Effect of NSAIDs on incidence of adenoma recurrence in randomised placebo controlled trials.

Reference	Patient group	N	Treatment (/day)	Incidence of recurrence	Relative risk (95% CI)
Baron ⁴³	Previous adenoma	1121	Placebo	47 %	
			81 mg aspirin	38 %	0.81(0.69-0.96)
			325 mg aspirin	45 %	0.96 (0.81-1.13)
Sandler ⁴²	Previous CRC	635	Placebo	27 %	
			325 mg aspirin	17 %	0.65 (0.46-0.91)
Benamouzig ⁴¹	Previous adenoma	272	Placebo	41 %	
			160 mg aspirin	35%	0.85 (0.57-1.26)
			300 mg aspirin	25%	0.61 (0.37-0.99)
Giardiello ⁴⁰	APC mutation carriers, phenotypically unaffected	41	Placebo	55%	
			150–300 mg sulindac	43%	0.52 (-0.29-2.73)

NSAIDS = non-steroidal anti-inflammatory drugs, APC = adenomatous polyposis coli, CI = confidence interval, N = number of patients, CRC = colorectal cancer.

PPAR γ ligands

The thiazolidine class of well established oral antidiabetic drugs, which include troglitazone, rosiglitazone and pioglitazone, are selective ligands for peroxisome proliferator-activated receptor γ (PPAR γ) ⁵⁰. PPAR γ is a ligand dependent transcription factor which can bind to specific response elements in the DNA to activate target genes ⁵¹. Mechanisms through which PPAR γ ligands may exert their effects include modulation of the oncogenic Wnt pathway, inhibition of NF κ B, modulation of cell cycle control components and changes in the expression of pro- and anti apoptotic proteins (Fig. 2). Wnt pathway activation, due to oncogenic Wnt signalling or mutations in *APC* or *β -catenin*, leads to stabilisation of β -catenin and its translocation to the nucleus ⁵². In the nucleus β -catenin binds to transcription factors which regulate genes involved in the regulation of proliferation and apoptosis ⁵². PPAR γ activation causes a decrease in β -catenin expression in adipocytes *in vitro* and in normal intestinal

mucosa in mice⁵³. The precise effect of PPAR γ ligands on β -catenin levels is unknown, but it has been suggested that PPAR γ activity controls the levels of known regulators of β -catenin, such as APC, axin and glycogen synthetase kinase 3 beta⁵⁴. In cultured human monocytes PPAR γ inhibits NF κ B activation thus influencing the transcription of both survival and apoptosis related genes⁵⁵. PPAR γ activation induced the activation of the pro-apoptotic caspase-3 protein in human liver cancer cell lines and a reduction in anti-apoptotic Bcl-2 and Bcl-XL protein levels in human colon and gastric cancer cell lines respectively⁵⁶⁻⁵⁸. In human liver cancer cells PPAR γ ligands influence cell-cycle control through modulation of cell-cycle control proteins^{59,60}. Activating *Ras* mutations, which occur in approximately 50% of colorectal tumours, inhibit PPAR γ transcriptional activity⁶¹⁻⁶³. This could, however, be overcome by simultaneously inhibiting Ras-activated pathways, as has been demonstrated in breast cancer⁶⁴. Evidence that PPAR γ ligands also work through PPAR γ independent mechanisms include the different effects of the various thiazolidines and the fact that the concentrations of PPAR γ ligands found to be effective in cancer cell line models (1×10^{-6} to 5×10^{-5} M) are several logs higher than those needed for modulation of PPAR γ target genes⁶⁵.

A number of studies have suggested that PPAR γ ligands and NSAIDs can interact and/or have effect on common pathways. Firstly, upregulation of PPAR γ expression by NSAIDs has been demonstrated in several tumour cell lines, indicating that simultaneous treatment with NSAIDs could enhance the effects of PPAR γ ligands^{66,67}. Secondly, a number of NSAIDs, including indomethacin, have been shown to behave as PPAR γ ligands⁶⁸. The antineoplastic effects of the NSAID indomethacin *in vitro*, however, do not seem to be mediated through PPAR γ ^{69,70}. Thirdly, the COX-2 promoter contains a peroxisome proliferator response element that can bind PPAR γ ligands⁷¹. Lastly, PPAR γ ligands inhibit the expression of tumour necrosis factor α (TNF- α)⁷². Since TNF- α is a strong inducer of NF κ B dependent COX-2 expression, PPAR γ ligands could reduce COX-2 levels through reductions in TNF- α ⁷³. Pioglitazone has been shown to protect against aspirin-induced gastric mucosal injury and to accelerate gastric ulcer healing in rats, possibly through this inhibition of tumour necrosis factor α expression⁷⁴. PPAR γ ligands can, therefore, induce two opposing effects on COX-2 expression. The first is the induction of COX-2 via the PPAR responsive element in the COX-2 promoter and the second is the inhibition of TNF- α -induced NF κ B activation and thus inhibition of COX-2 expression (Fig. 2)⁷⁵.

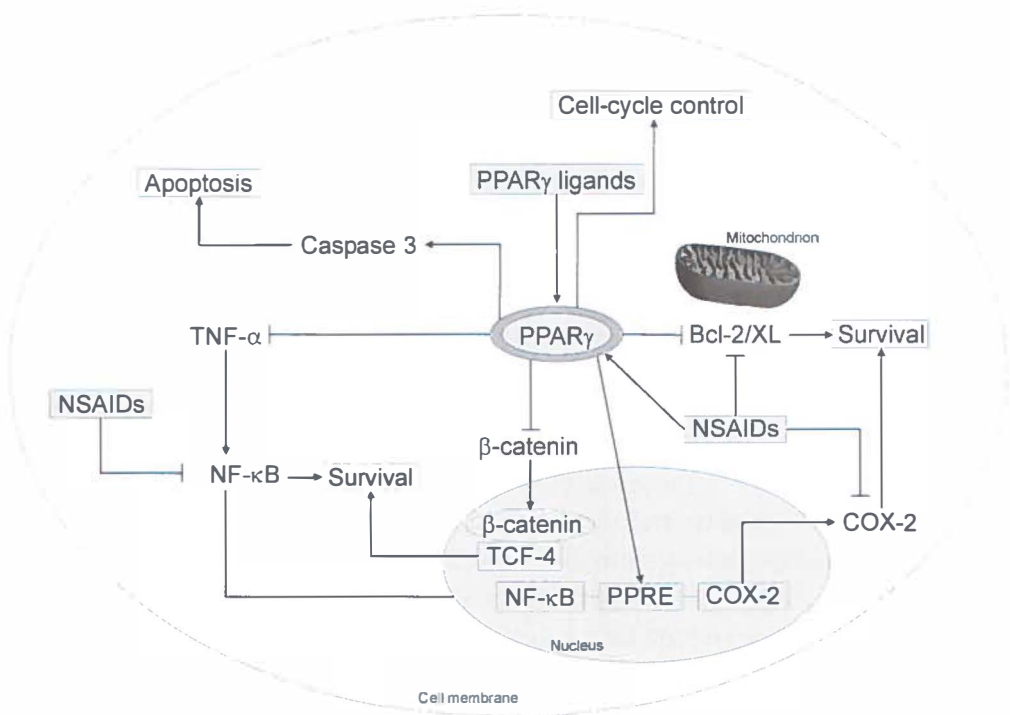


Figure 2: Molecular mechanisms of effects of PPAR γ ligands and possible mechanisms of interaction with NSAIDs. NSAIDs = non-steroidal anti-inflammatory drugs, PPAR γ = peroxisome proliferator-activated receptor γ , PPRE = peroxisome proliferator response element, COX = cyclo-oxygenase, NF κ B = nuclear factor kappa B, TCF = T-cell factor. Stimulates \longrightarrow Inhibits \dashv

This could explain the fact that PPAR γ ligands can induce dose dependent COX-2 expression in human colon cancer cell lines after 10-12 hours of treatment but that after 24 hours PPAR γ ligands down-regulate COX-2 expression^{71,76}. These opposing effects on COX-2 expression seem to occur at different PPAR γ ligand concentrations and at different times after treatment. This makes the combination of PPAR γ ligands and NSAIDs interesting because, besides their individual efficacy and modulation of common pathways, inhibition of COX-2 by NSAIDs could counteract any negative effects of increased COX-2 expression due to PPAR γ ligand treatment. So far, there have been no reports of simultaneous treatment of cultured colon cancer cells with NSAIDs and PPAR γ ligands.

Approximately 8% of primary colorectal carcinomas contain a loss of function mutation in one PPAR γ allele supporting a role for PPAR γ as a tumour suppressor⁷⁷. Seemingly in contrast to this, PPAR γ protein levels are generally higher in human colorectal carcinomas as compared to normal colon epithelium⁷⁸. In rodents, troglitazone, pioglitazone and rosiglitazone all reduce the formation of chemically induced colon tumours^{79,80}. It has been suggested that this protective effect is due to reductions in β -catenin levels by the PPAR γ ligands. It is thought that PPAR γ ligands do not affect β -catenin levels in tumours once they are established and/or the Wnt pathway has been activated. This theory is supported by the fact that although PPAR γ ligands reduce β -catenin levels in normal colon tissue in mice, no difference was seen in β -catenin staining between tumours from PPAR γ treated and untreated mice⁷⁹. Further support comes from a study in mice with wild-type APC in which more polyps and higher β -catenin levels were seen when only one functional PPAR γ allele was present compared to the situation in PPAR γ wild type mice. However, in mice with only one functional APC allele (APC^{+/-1638} mice) the PPAR γ status did not affect polyp number or β -catenin levels⁵⁴. Further studies in APC deficient mouse models show conflicting results regarding the effects of PPAR γ ligand treatment (Table 3)⁸¹⁻⁸⁴. Compared to controls, troglitazone treatment caused increases in polyp number in Apc^{mn/+} mice, but pioglitazone caused a reduction in polyp number in the same model⁸²⁻⁸⁴. In a different model, APC^{+/-1309} mice, pioglitazone treatment also caused a decrease in polyp number⁸¹.

Table 3: Results of PPAR γ ligand treatment in mouse models for FAP

Author	Model	Drug	Duration	Dietary dose	Result (polyp number)	
Saez ⁸⁴	APC ^{+/-min}	Troglitazone	5 weeks	2000 ppm	Increase	P<0.02
Lefebvre ⁸³	APC ^{+/-min}	Troglitazone	8 weeks	1500 ppm	Increase	P<0.05
Niho ⁸¹	APC ^{+/-1309}	Pioglitazone	6 weeks	100-200 ppm	Decrease	P<0.05
Niho ⁸²	APC ^{+/-min}	Pioglitazone	14 weeks	100-1600 ppm	Decrease	P<0.01

PPAR γ = peroxisome proliferator-activated receptor, FAP = familial adenomatous polyposis, ppm = parts per million.

In summary, although all PPAR γ ligands are able to prevent chemically induced colon tumours in rodents, this is not always the case for prevention of intestinal tumours in *Apc*^{min/+} mice. It has been suggested that inhibition of colorectal polyps in *Apc*^{min/+} mice, which have an activated Wnt pathway due to heterozygous loss of *APC*, is a PPAR γ independent effect of pioglitazone, but not of troglitazone⁸². In colon cancer cell lines *APC* status does not seem to influence the effect of PPAR γ ligands. There are many pathways activated in carcinomas, on which PPAR γ ligands can exert effects, which are not yet activated in adenomas and this could explain the fact that all PPAR γ ligands seem to be effective in colon cancer cell lines while there are differences in effectiveness in adenoma prevention⁸⁵. Exposure of human colon cancer cell lines to PPAR γ ligands can result in cell growth arrest, induction of cell differentiation and increased apoptosis, irrespective of *APC* status^{77,86}. In nude mice, troglitazone inhibits growth of tumours derived from human colon cancer cells⁷⁷.

Adipocytes express PPAR γ at high levels and in a phase II clinical trial in 3 patients with advanced liposarcoma, treatment with troglitazone was shown to induce biochemical and histological differentiation and a reduction in proliferation markers⁸⁷. In another phase II study, treatment of 41 prostate cancer patients with troglitazone for 18 weeks led to a stabilisation of prostate specific antigen in 20% of patients⁸⁸. In a phase II trial in 25 patients with chemotherapy resistant metastatic colorectal cancer, troglitazone was well tolerated but no objective tumour response was observed⁸⁹. Thiazolidines are already widely used clinically for the treatment of type 2 diabetes mellitus with minimal toxicity. Side effects include an increased plasma volume leading to oedema, slight reductions in haemoglobin and haematocrit, weight gain and alterations in plasma lipid profiles⁹⁰. Despite weight gain and increased low density lipoprotein (LDL) cholesterol, intra-abdominal fat deposition, leading to increased cardiovascular risk, does not occur⁹⁰. Troglitazone is no longer available due to rare, but severe, hepatotoxicity caused by the troglitazone metabolite, quinone^{91,92}. This problem has not been reported for other thiazolidines.

In summary, although the results of clinical trials designed to demonstrate anti-tumour effect showed no objective response, there is some preclinical data to support testing of PPAR γ ligands in a different setting, namely in the chemoprevention of colorectal cancer. The low toxicity of- and extensive clinical experience with PPAR γ ligands make these drugs especially attractive.

Furthermore, the combination with NSAIDs is interesting since PPAR γ ligands could increase effectivity while possibly reducing the gastrointestinal toxicity of NSAIDs. However, the reasons for contradictory pre-clinical results regarding the efficacy of PPAR γ ligands need to be firmly established before clinical chemoprevention trials can be considered.

Cholesterol synthesis inhibitors

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (HRIs), also known as statins, are widely used to reduce serum cholesterol in the treatment of hypercholesterolemia. HRIs reduce cholesterol synthesis by inhibiting the rate-limiting enzyme HMG-CoA and thereby preventing the formation of mevalonate from HMG-CoA. The end products of this pathway are required for a number of essential cellular functions including membrane integrity and (glyco)protein synthesis. Furthermore, they are involved in cell signalling cascades and carcinogenesis⁹³. Statins induce apoptosis and inhibit proliferation in tumour derived cells, including colon cancer cells⁹³⁻⁹⁵. Mechanisms include upregulation of pro-apoptotic proteins, induction of cell-cycle arrest and inhibition of metastasis (Fig. 3).

In a leukemic cell line, statin induced apoptosis seems to employ the mitochondrial pathway, leading to cytochrome-c release, caspase-3 activation and PARP cleavage to induce apoptosis⁹⁶. Lovastatin treatment leads to upregulation of caspase-7 in prostate cancer cell lines⁹⁷. Statins downregulate the anti-apoptotic Bcl-2 and upregulate the pro-apoptotic protein Bax in cultured colon cancer cells⁹⁸. Interestingly, interactions with NSAIDs have also been described. Lovastatin augments sulindac-induced apoptosis in several colon cancer cell lines⁹⁴. Addition of down-stream products of cholesterol reverses the lovastatin augmentation of sulindac-induced apoptosis indicating that the effect is due to inhibition of HMG-CoA⁹⁴.

This effect could be mediated through modulation of the Wnt pathway since lovastatin alters the function of rho GTPase proteins which are involved in regulation of β -catenin levels⁹⁴. Lovastatin also enhanced celecoxib-induced apoptosis and caspase-3 activation in cultured colon cancer cells⁹⁹. Apart from apoptosis induction, the exposure of transformed cells to statins *in vitro* also leads to G1 growth arrest^{100,101}. This response is p53 independent and is mediated by a downregulation of cyclin dependent kinase (CDK) activity and

upregulation of CDK inhibitors p21 and p27^{95,101,101}. Statins have been shown to block metastasis at the level of cell attachment, migration and invasion in *in vitro* and rodent models⁹³.

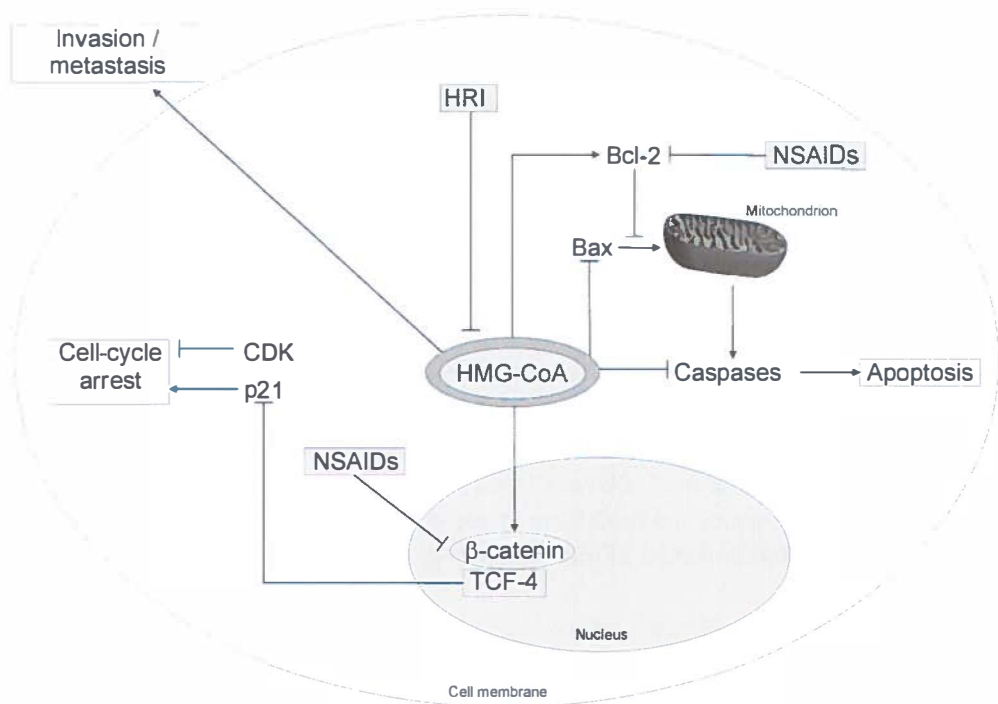


Figure 3: Molecular mechanisms of effects of HRIs and possible mechanisms of interaction with NSAIDs. NSAIDs = non-steroidal anti-inflammatory drugs, HRI = 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, TCF = T-cell factor, CDK = cyclin dependent kinase. Stimulates →. Inhibits —|.

Pravastatin and simvastatin reduce carcinogen-induced colon cancers in rodent models by 50-60%^{101,102}. Furthermore, lovastatin leads to 30% reduction in the appearance of premalignant aberrant crypt foci in rodent models with carcinogen induced colon cancer^{94,103}. In two clinical trials designed to study the effects of the HRIs pravastatin (4,159 patients, 83% concomitant NSAID use in both arms) and simvastatin (4,444 patients, 37% concomitant NSAID use in both arms) on coronary artery disease, reductions of 43% and 19% respectively in the number of new cases of colorectal cancer during the 5 year follow-up period were observed in the statin treated groups^{104,105}. Another trial (6,605 patients, 17% concomitant NSAID use in both arms),

however, did not find a reduction in colon cancer incidence after 5 years of lovastatin use ¹⁰⁶. Agarwal *et al.* suggest that chemopreventive effect of HRIs increases with NSAID use based on the reduction in colon cancer found in relation to the percentage of patients using NSAIDs in these three clinical trials with HRIs ⁹⁴. In a recent population based case-control study of incident colorectal cancer, use of HMG-CoA reductase inhibitors was associated with a 51% reduction in the risk of colorectal cancer and this reduction in risk was independent of NSAID use ¹⁰⁷. In phase I trials with lovastatin tested as an anti-cancer drug, it was possible to reach plasma concentrations which inhibit growth *in vitro* while toxicity was minimal ^{108,109}. In a phase II trial, lovastatin did not give objective tumour response in 16 patients with gastric adenocarcinoma ¹¹⁰. In one patient with relapsed acute myelogenous leukemia lovastatin treatment decreased blast count during, and up to 3 months after, treatment ¹¹¹. In a randomised controlled trial with pravastatin in addition to standard treatment in 83 patients with irresectable hepatocellular carcinoma, survival was longer in the treatment group compared to the control group (18 months vs. 9 months) ¹¹². HRIs have been in use for more than 15 years with no significant toxicity even when used for long periods. In the clinical trials with lovastatin in cancer patients toxicity was minimal and consisted of gastrointestinal and musculoskeletal complaints ⁹³.

In summary, long-term HRI use is associated with a risk reduction for colorectal cancer, especially in patient groups with a high percentage of NSAID users. *In vitro* and preclinical *in vivo* studies confirm the anti-tumour effect of statins in colorectal cancer. These drugs are interesting for colorectal cancer chemoprevention since they can be given orally for long periods of time without significant toxicity. Further preclinical *in vivo* and clinical chemoprevention trials are necessary in the near future to determine the effect of cholesterol synthesis inhibitors, especially in combination with NSAIDs, in the preventive setting.

Inhibitors of the epidermal growth factor family

The epidermal growth factor receptor (EGFR) family consists of four closely related membrane receptors: human epidermal growth factor receptor-1 (EGFR, HER-1 or ErbB-1), HER-2 (neu or ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4) ¹¹³. Intrinsic stimulatory ligands include epidermal growth factor (EGF) and transforming growth factor α . Ligand binding causes homo- or heterodimerisation of two receptors, activation of intrinsic protein kinase activity

and activation of different intra-cellular signalling routes including the Ras and Akt pathways (Fig. 4) ^{114,115}. Activation of the Ras pathway influences processes including proliferation and apoptosis ¹¹⁵. Activation of the protein kinase Akt relays survival signals by regulation of cell cycle progression and inactivation of targets including the pro-apoptotic Bad and caspase-9 ¹¹⁶⁻¹¹⁸. Activation of growth factor receptors furthermore contributes to modulation of cell migration, adhesion and angiogenesis ¹¹⁹.

Reported immunohistochemical expression of EGF receptors in human colorectal carcinomas vary, possibly due to differences in patient groups, antibodies and definitions for evaluation used ¹²⁰. Most studies indicate that immunohistochemical overexpression of EGFR, which is probably not caused by gene amplification, occurs in approximately 50% of colorectal tumours ^{121,122}. HER-2 overexpression is secondary to gene amplification and seen in just 2.4% of colorectal tumours, although immunohistochemical studies report much higher percentages ¹²⁰. Differences in expression of EGFR and HER-2 between adenomas and carcinomas have been reported. There is, however, conflicting evidence regarding the question whether adenomas or carcinomas have higher expression ^{123,124}. Overexpression of COX-1 and COX-2 in human colon carcinoma cell lines has been shown to increase HER-2 mRNA expression ¹²⁵. Immunohistochemical HER-2 expression was reduced in rectal mucosa of patients treated with low dose sulindac ¹²⁶. In human colon cancer cell lines EGFR activation stimulates COX-2 expression and inhibitors of EGFR and HER-2 decrease COX-2 expression ^{127,128}. These studies indicate that EGF receptors and COX-2 levels are linked in a positive feedback loop during colorectal cancer development ¹⁷.

EGF pathways can be inhibited by neutralising the natural ligands, blocking the EGFR or HER-2 or inhibiting the EGFR tyrosine kinase activity which initiates the intracellular pathways. Antagonistic antibodies against EGFR cause decreases in levels of the anti-apoptotic Bcl-2 and increases in levels of pro-apoptotic proteins such as Bax and various caspases (Fig. 4) ¹²⁹⁻¹³¹. EGF signalling inhibition causes cell cycle arrest, decreased proliferation and induction of apoptosis in colon carcinoma cell lines *in vitro* ¹³²⁻¹³⁶. EGF signalling inhibition also has an effect on angiogenesis. This effect is mediated through a decrease in vascular endothelial growth factor, a decrease in blood vessel formation and the induction of apoptosis in endothelial cells ^{137,138}. Furthermore, inhibition of invasion and metastasis *in vivo* and a decrease in the migration of cells *in vitro* can be initiated by inhibition of EGF signalling ¹³⁸.

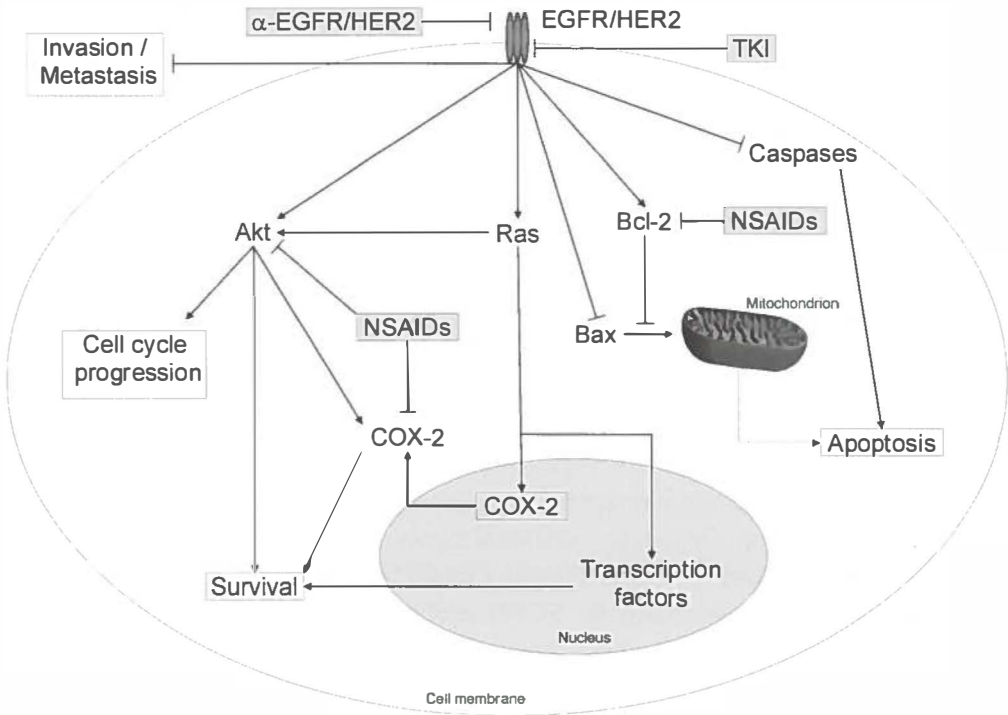


Figure 4: Molecular mechanisms of effects of EGF inhibition and possible mechanisms of interaction with NSAIDs. NSAIDs = non-steroidal anti-inflammatory drugs, COX = cyclo-oxygenase, EGF = epidermal growth factor, EGFR = epidermal growth factor receptor, TKI = tyrosine kinase inhibitor, α = blocking antibody. Stimulates \longrightarrow
Inhibits \longrightarrow .

The *Ras* genes are important down-stream mediators of EGF-transduced signals, however, *K-ras* is mutated in approximately 50% of colorectal tumours. Interestingly, *K-ras* mutation status did not affect the sensitivity of lung cancer cell lines to inhibition of EGF signalling¹³⁹. Interactions between EGF signalling and NSAID mediated effects have been described. Prostaglandins transactivate the EGFR receptor in human colon cancer cell lines and sulindac blocks EGF signalling^{140,141}. Combination therapy with celecoxib and a HER-2 antibody had an additive effect that resulted in almost complete inhibition of human HER-2 positive colon tumour growth *in vitro* and was more effective than either agent alone¹⁴².

Anti-EGFR monoclonal antibody treatment and also EGFR tyrosine kinase inhibitors (EKIs) can cause dose dependent growth inhibition of colon carcinoma xenografts¹⁴³⁻¹⁴⁵. $Apc^{min/+}$ mice lacking a functional EGFR receptor developed 90% less polyps than EGFR expressing $Apc^{min/+}$ mice, indicating that inhibition of EGFR could reduce polyp numbers¹⁴⁴. This was confirmed in regular $Apc^{min/+}$ mice where treatment with an EKI resulted in a reduction in intestinal polyp numbers of up to 50%¹⁴⁶. Another chemically distinct specific tyrosine kinase inhibitor, however, did not give this result^{146,147}. The combination of an irreversible EKI and the NSAID sulindac prevented polyp development in $Apc^{min/+}$ mice more effectively than either drug alone and made it possible to reduce the dose of sulindac needed for effective polyp prevention¹⁴⁶. In phase II trials the EGFR monoclonal antibody cetuximab, alone or in combination with chemotherapy, produced objective responses in up to 17% of patients with refractory, EGFR positive colorectal cancer^{148,149}. Based on the phase II trials and a randomised, controlled trial in 329 patients with refractory, EGFR-expressing metastatic colorectal cancer, cetuximab has recently been registered for use in disseminated colorectal cancer in combination with irinotecan (<http://www.fda.gov>). EGFR antibodies are generally well tolerated and in combination with chemotherapy in colorectal cancer levels of toxicity were acceptable^{148,150}. The major reported side-effects include fever, nausea, elevation of liver enzymes and allergic and acneiform skin reactions¹⁵¹. Trastuzumab, an anti-HER-2 monoclonal antibody, has been registered as an anti-cancer drug for the treatment of metastatic breast cancer¹⁵². Cardiac toxicity induced by anti-HER-2 antibody therapy is the side-effect of greatest clinical concern¹⁵³. Considering the low expression of HER-2 in colon cancer and the cardiac toxicity, HER-2 targeted therapy does not seem feasible in the prevention of colorectal cancer. Based on randomised phase II trials the EKI gefitinib has recently been registered in several countries for use in pre-treated advanced non-small-cell-lung-cancer (NSCLC) patients¹⁵⁴. In this setting there is a tumour response rate of approximately 20 %. It has recently been shown that response is associated with mutations in EGFR and this indicates that screening for these mutations could identify patients who will respond to gefitinib^{155,156}. In a recent patient, double blind, placebo controlled phase III trial, in 731 patients, the EKI erlotinib was more effective than placebo in the treatment of patients with advanced or metastatic NSCLC following the failure of first or second line chemotherapy as measured in terms of both survival and tumour response (www.drugs.com/NDA/tarceva_040629.html). In phase II trials with the EKIs gefitinib (27 patients) and erlotinib (25 patients) in patients with metastatic colorectal cancer there were, however, no responders¹⁵⁷. The frequency and role of EGFR mutations in colorectal cancer is not yet clear. In

studies with EKIs the most frequent adverse events were nausea, vomiting, headache, fatigue, an acneiform rash and diarrhoea. These side-effects were often manageable by applying dose reduction and using supportive medication such as anti-emetics ¹⁵¹.

In summary, *in vitro* and preclinical *in vivo* studies have shown effectivity of EGF signalling inhibition in colorectal cancer. Considering the high EGFR expression and low HER-2 expression in colorectal cancer, therapies targeting the EGFR are appealing. Clinically, especially EGFR antibodies are of interest since these drugs have been registered for colorectal cancer, whereas phase II trial results of EKI therapy in colorectal cancer are disappointing. There are indications that combining these drugs with NSAIDs could lead to enhanced effects. These results are interesting with respect to possibilities for the chemoprevention of colorectal cancer. However, high costs make these drugs less suited for long-term use at this moment. Furthermore, the long-term toxicity profiles of EGF signalling inhibitors as well as the efficacy in colorectal cancer need to be further evaluated to determine the feasibility in the preventive setting.

TRAIL receptor ligands

TNF Related Apoptosis Inducing Ligand (TRAIL) is a member of the TNF family which also includes TNF and Fas-ligand. It induces apoptosis in a wide variety of cancer cell lines. Unlike TNF and Fas-ligand, TRAIL has little or no detectable cytotoxic effect on normal cells *in vitro* and *in vivo* and therefore has potential as an anti-cancer agent ¹⁵⁸. Modulation of TRAIL induced apoptosis by chemotherapeutic agents in colon cancer cell lines has been described ¹⁵⁹. Furthermore, TRAIL potentiates NSAID induced apoptosis *in vitro* ^{160,161}.

Four membrane-bound receptors for TRAIL have been identified: two cell death-inducing receptors (DR4 and DR5) and two decoy receptors (DcR1 and DcR2) ¹⁶². The intracellular segments of DR4 and DR5 contain death domains required for TRAIL-mediated apoptotic cell death ¹⁶³. In contrast, DcR1 lacks the intracellular death domain and DcR2 contains a truncated death domain and therefore neither receptor can induce apoptosis. TRAIL induces apoptosis by binding to DR4 or DR5. COX-2 overexpression was shown to reduce DR5 expression in association with attenuated TRAIL induced apoptosis ¹⁶⁴.

Sulindac sulphide treatment restored DR5 expression and, when combined with TRAIL, reduced cell survival more than either drug alone ¹⁶⁴.

In colon cancer cell lines, sulindac sulphide and the NSAID SC-236 upregulated DR5 expression in a COX-2 independent manner (Fig. 5) ^{160,165}. Therefore, it is likely that DR5 upregulation by NSAIDs is mediated through both COX-dependent and COX-independent effects. TRAIL binding to DR4 or DR5 induces the recruitment of specific cytoplasmic proteins to the intracellular death domain of the receptor thus forming a death inducing signalling complex (Fig. 5). Caspase-8 is recruited to this complex and can transduce the apoptotic signal to down-stream executioner caspases ¹⁶⁶. This is known as the extrinsic pathway. Sulindac sulphide has been shown to potentiate TRAIL induced activation of caspase-8 in various colon cancer cell lines ^{160,165}. TRAIL induced apoptosis can also occur through the caspase-8 activated intrinsic (mitochondrial) cellular pathway. Caspase-8 activation causes activation of Bid which influences the balance of pro- and anti-apoptotic proteins belonging to the Bcl-2 family resulting in cytochrome-c release from mitochondria ¹⁶⁷. This in turn promotes the activation of caspase-9, which activates downstream executioner caspases. Bax is a pro-apoptotic member of the Bcl-2 family which is thought to be important in TRAIL resistance. The effectiveness of TRAIL induced apoptosis is lost in Bax deficient cells and TRAIL treatment leads to selection for Bax mutations in microsatellite unstable colon cancer cells ¹⁶⁸. This is relevant because *Bax* is mutated in up to 50% of HNPCC and in a subset of sporadic, microsatellite instable, colorectal cancers ^{160,169,170}. The combination of NSAIDs with TRAIL has, in some settings, been shown to overcome Bax deficiency and induce apoptosis ^{160,160}. Inhibitors of TRAIL induced apoptosis include FLICE inhibitory protein (FLIP), inhibitor of apoptosis proteins (IAPs) and the anti-apoptotic members of the Bcl-2 family of proteins (Fig. 5) ¹⁷¹.

The influence of NSAIDs on these inhibitors may be another mechanism for the positive effects of these drugs on TRAIL induced apoptosis. The reduction in expression of the anti-apoptotic protein Bcl-X_L by sulindac sulphide treatment augmented TRAIL induced apoptosis ¹⁶⁵. Treatment of colon cancer cells with sulindac attenuated the expression of the IAP family member survivin ¹⁷². NSAIDs can also inhibit NFκB which is involved in the expression of several inhibitors of TRAIL induced apoptosis such as FLIP, the IAPs and Bcl-2 ¹⁷³⁻¹⁷⁵.

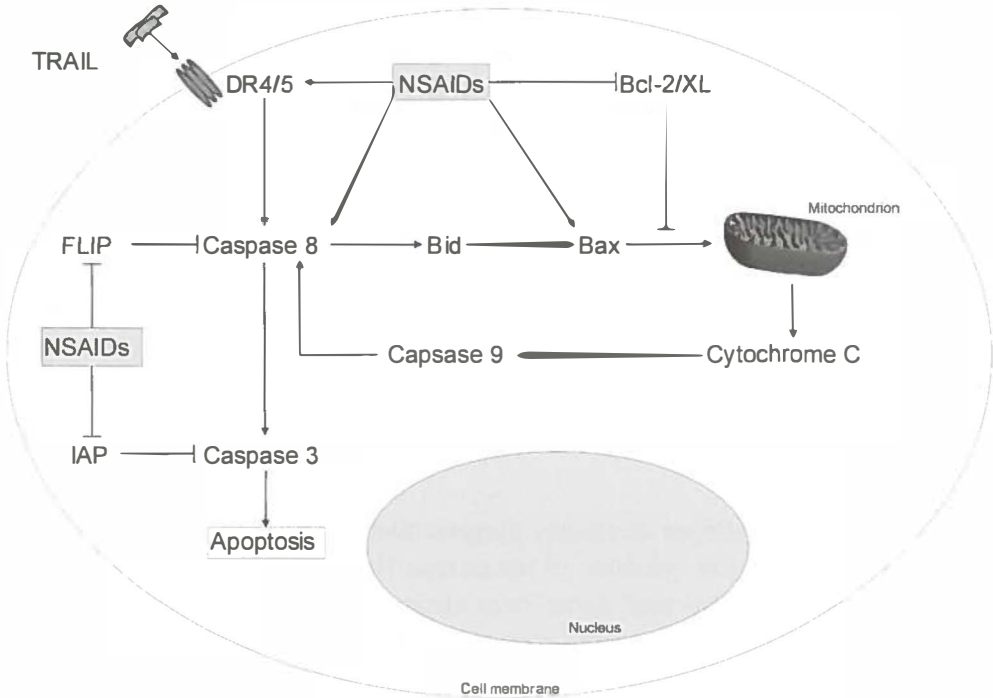


Figure 5: Molecular mechanisms that mediate TRAIL-induced apoptosis and mechanisms of interaction with NSAIDs. NSAIDs = non-steroidal anti-inflammatory drugs, TRAIL = TNF-related apoptosis inducing ligand, DR = death receptor, FLIP = FLICE-like inhibitory protein, IAP = inhibitor of apoptosis. Stimulates \rightarrow
Inhibits \mid

Colorectal adenomas and carcinomas have stronger immunohistochemical expression of the pro-apoptotic receptors DR4 and DR5 in comparison with normal colon epithelium in both sporadic and HNPCC cases¹⁷⁶⁻¹⁷⁸. Recombinant human TRAIL (rhTRAIL) induces apoptosis in human colon cancer cell lines¹⁷⁹. RhTRAIL alone and in combination with chemotherapeutic agents induced apoptosis in human colon cancer cell line xenografts in immune deficient mice¹⁸⁰. DR4 and DR5 agonistic monoclonal antibodies have also been shown to induce apoptosis in human cancer cells *in vitro* and *in vivo*¹⁸¹⁻¹⁸³. Different forms of rhTRAIL have been tested and it is now possible to produce native rhTRAIL without added exogenous sequences¹⁷⁹. This is relevant because versions of rhTRAIL with exogenous sequences have been shown to induce toxicity in cultured human hepatocytes, cultured primary human aorta and pulmonary smooth muscle cells and human brain tissue

slices^{184,185}. In contrast, native rhTRAIL was non-toxic for cultured hepatocytes¹⁸⁶. The expression of the TRAIL receptors in chimpanzees is comparable to that in humans¹⁸⁷. Human TRAIL binds to cynomolgous monkey receptors, which themselves are highly conserved, with an affinity and homology comparable to human receptors and native rhTRAIL did not cause detectable toxicity in cynomolgus monkeys or chimpanzees^{179,188}. At high concentrations, humanised monoclonal agonistic antibodies against DR4 and DR5 can induce apoptosis in cultured human hepatocytes¹⁸⁹. Phase I studies with these antibodies are currently in progress (<http://www.hgsi.com/products/>) and have not been terminated due to severe toxicities. This suggests that rhTRAIL is also non-toxic for normal tissue in humans. It must be noted that receptor upregulation by NSAIDs in normal tissues might increase the toxicity of rhTRAIL.

In summary, a number of studies suggest that TRAIL and NSAIDs may act synergistically in the induction of apoptosis. The combination of these drugs seems more effective than either drug alone and, importantly, appears to be effective in Bax deficient cells. We speculate that the future use of TRAIL receptor ligands may not be limited to malignant lesions but may also become applicable to premalignant lesions such as colorectal adenomas when combined with NSAIDs. This is, however, only possible after effectivity and long-term toxicity of TRAIL have been intensely studied in the treatment setting. The dosage form and dosage schedule would need to be adapted for the preventive setting since TRAIL has a short half-life and will initially be delivered intravenously. At this moment, long-term administration of current TRAIL-receptor agonists is unsuitable for use in the preventive setting due the high costs. In analogy with the development of synthetic EGF signalling inhibitors, another possibility is the development of synthetic molecules which activate TRAIL-induced apoptosis, that have a longer half-life and can be given orally. Finally, it is necessary to determine the functionality of the TRAIL receptors and the influence of NSAIDs on TRAIL-induced apoptosis in colorectal adenomas and carcinomas, as well as the effect on normal colonic epithelium, before their use in the preventive setting can be further contemplated.

Conclusion

Although the role of NSAIDs as chemopreventive agents in colorectal cancer is well established they do not offer complete protection against adenoma development. At this moment the advantages are only regarded to outweigh the disadvantages in patients with FAP. More accurate identification of high risk groups is necessary together with the development of combinations of drugs which could increase the chemopreventive effect of NSAIDs. These drugs should, furthermore, have minimal toxicity levels and be available in oral form. There is increasing interest in agents that can target specific signalling pathways in malignant or premalignant cells. It must be noted that although the use of adenomas as surrogate intermediate markers for colorectal cancer is widely accepted and necessary to limit trial size and length, there are some limitations to this approach. Only a subset of adenomas, especially larger ones, those with a villous component and highly dysplastic adenomas, eventually progress to carcinoma. Furthermore, mechanisms involved in adenoma development differ from those involved in adenoma-to-cancer progression. It is important to establish additional (biological) markers as surrogate end-points which correlate closely to disease progression rather than polyp development to evaluate potential chemopreventive regimen^{44,190}. Based on the available literature we hypothesise that the following combinations deserve further attention in the chemoprevention of colorectal cancer. Firstly, NSAIDs could be combined with PPAR γ ligands which have been shown to inhibit the development of colorectal cancer *in vitro* and *in vivo* and, importantly, are currently widely prescribed and well tolerated in patients with diabetes mellitus. Secondly, long term statin use is associated with a risk reduction for colorectal cancer and *in vitro* and *in vivo* studies with HRIs have confirmed the anti-tumour effects in colorectal cancer. Thirdly, EGF signalling inhibitors have recently been shown to potentiate the chemopreventive effects of NSAIDs *in vitro* and *in vivo*. EGF signalling inhibitors have reached phase III clinical trials in lung and colon cancer and oral versions are being developed. Finally, recent evidence that TRAIL potentiates NSAID induced apoptosis *in vitro* forms a rationale to further explore the combination of TRAIL and NSAIDs as a novel chemoprevention strategy in *in vivo* models. It will, however, be a long time before TRAIL and EGF signalling inhibitors could, potentially, be used in the preventive setting considering the early stages of development of these drugs and the current high costs. Statins and PPAR γ ligands could be developed for colorectal cancer chemoprevention more rapidly since there is considerable experience with these drugs in different clinical settings and the costs are

relatively low. The way forward is to further clarify and understand the mechanisms of action of NSAIDs in combination with these drugs *in vitro* and *in vivo* and to examine the effectiveness of these chemopreventive regimen *in vivo*.

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Chapter 3

TRAIL induces apoptosis in human colorectal adenoma cell lines and human colorectal adenomas

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Abstract

Purpose: Recombinant human (rh) tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potential new anti-cancer drug which can induce apoptosis in colorectal cancer cell lines. The aim of this study was to investigate whether it is possible to induce apoptosis in human adenoma cell lines and human adenomas using rhTRAIL.

Experimental Design: Two human adenoma cell lines were exposed to rhTRAIL, 0.1 µg/ml for 5 hours. Apoptosis and caspase activation in cell lines were evaluated using immunocytochemistry, fluorimetric caspase assays and Western blotting. Short-term explant cultures were established from freshly removed human adenomas (n=38) and biopsies of normal colon epithelium (n=15) and these were incubated for 5 hours in the presence or absence of 1 µg/ml rhTRAIL. Apoptosis was determined in paraffin-embedded tissue using morphological criteria and cleaved caspase-3 staining.

Results: In the adenoma cell lines rhTRAIL induced up to 55% apoptosis. This coincided with caspase-8 and -3 activation and could be inhibited by a pan-caspase inhibitor. RhTRAIL induced caspase-dependent apoptosis in adenomas with high-grade dysplasia (n=21) compared to the paired untreated counterparts (apoptotic index 34% ± 5% vs. 17% ± 2%, mean ± SEM, p = 0.002), but not in adenomas with low-grade dysplasia (n=17) or in normal colon epithelium (n=15).

Conclusions: Colorectal adenoma cell lines and adenomas with high-grade dysplasia are sensitive to rhTRAIL-induced apoptosis whereas normal colon epithelium is not. This suggests the potential application of rhTRAIL in the treatment of adenomas with high-grade dysplasia.

Introduction

TRAIL is a member of the TNF-family of death receptor ligands which has been shown to induce apoptosis in a wide variety of cancer cell lines. Unlike the other TNF-family members, TNF- α and Fas-ligand, TRAIL has little or no detectable cytotoxic effect on normal cells in vitro and in vivo and therefore has potential as an anti-cancer agent ¹. Four membrane-bound receptors for TRAIL have been identified: two cell death-inducing receptors (DR4 and DR5) and two decoy receptors (DcR1 and DcR2) ². The intracellular segments of DR4 and DR5 contain death domains required for TRAIL-mediated apoptotic cell death ³. In contrast, DcR1 lacks the intracellular death domain and DcR2 contains a truncated death domain and therefore these receptors cannot induce apoptosis. TRAIL binds as a homotrimer to DR4 and/or DR5, inducing trimerization of these receptors, which leads to the formation of a death inducing signalling complex, activation of caspase-8 and -3 and eventually results in apoptosis ⁴. Recombinant human TRAIL (rhTRAIL) induces apoptosis in human colon cancer cell lines and also in human colon cancer cell line xenografts in mice ^{5,6}. Phase I studies with rhTRAIL and phase I-II studies with agonistic monoclonal antibodies against DR4 and DR5 are currently in progress in cancer patients.

Like carcinomas, colorectal adenomas have stronger immunohistochemical expression of the pro-apoptotic receptors DR4 and DR5 in comparison with normal colon epithelium, suggesting that adenomas could also be sensitive to rhTRAIL induced apoptosis ⁷⁻⁹. This is interesting since not only sporadic adenomas, but also adenomas from patients with hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) express DR4 and DR5 ⁹. This makes rhTRAIL a potential therapeutic agent for the treatment of colorectal adenomas as well as colorectal carcinomas. However, the TRAIL sensitivity of a pre-malignant lesion has never been demonstrated. Therefore, the next step is to establish whether the DR4 and DR5 receptors expressed in colorectal adenomas are functional and lead to TRAIL sensitivity.

In the present study we determined the sensitivity of human adenoma cell lines and freshly removed human colorectal adenomas and normal colon epithelium for rhTRAIL induced apoptosis.

Patients and methods

Cell culture

The human adenoma cell lines VACO-235 and VACO-330, derived from a villous and tubular adenoma respectively, were used (Kindly provided by J.K. Willson MD, Ireland Cancer Center, Cleveland, OH, USA)¹⁰. Early passages of both cell lines are non-tumourigenic in athymic mice, show anchorage-dependent growth in culture and exhibit features of well differentiated epithelial cells, including cell polarity, microvilli and junctional complexes¹⁰. The VACO 235 cell line is APC mutant at both alleles, has a mutant K-ras allele and wild type p53¹¹. Mutation status of the VACO-330 cell line has not been described. Early passages of the cell lines were used in all cases. The cell lines were maintained on rat tail collagen-coated plates in MEM supplemented with 2% foetal bovine serum, insulin, transferrin, selenium, epithelial growth factor and hydrocortisone (MEM2+ media) as previously described¹⁰. To compare the sensitivity of adenoma cell lines for rhTRAIL to that of colon cancer cell lines, the colon cancer cell line LOVO was used¹². Continuous incubation of the LOVO cell line with 0.04 µg/ml rhTRAIL for 96 hours resulted in 50% growth inhibition (data not shown) making this cell line intermediately sensitive in comparison with other human colon cancer cell lines such as SW948, Caco-2 and Colo320 that have been used in our laboratory¹³. The LOVO cell line was cultured in RPMI (Invitrogen Life Technologies, Breda, The Netherlands) supplemented with 10% foetal calf's serum.

Flow cytometry for membrane expression of TRAIL receptors

Analysis of TRAIL-receptor membrane expression (DR4, DR5, DcR1, DcR2) was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL, USA) and cells were stained as described earlier¹³. Membrane receptor expression is shown as mean fluorescence intensity of all analyzed cells. All experiments were performed at least three times.

Quantification of apoptosis in cell lines

Immunocytochemistry was used to quantify apoptosis in the adenoma and carcinoma cell lines. Cells were incubated with rhTRAIL 0.1 µg/ml and/or 50 µM of the broad spectrum caspase inhibitor zVAD-fmk (Calbiochem, Breda, The Netherlands) for 5 hours at 37°C before cytopspins were made. RhTRAIL was produced non-commercially in co-operation with IQ-Corporation (Groningen, the Netherlands) as described previously¹⁴. Cytopspins were fixed and permeabilized before incubation with cleaved caspase-3 antibody (1:100,

Cell Signalling Technology, Leusden, The Netherlands) followed by incubation with appropriate secondary and tertiary antibodies. The staining was visualized using 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Cleaved caspase-3 positivity was identified as brown cytoplasmic staining. At least 300 cells were counted and the percentage of apoptotic cells was determined. All experiments were performed in triplicate.

Protein expression after apoptosis induction by rhTRAIL

Adenoma cells were incubated with rhTRAIL 0.1 µg/ml for 5 hours at 37 °C. Preparation of protein lysates and Western blot analysis was performed as described previously¹³. The following antibodies were used: rabbit-anti-cleaved caspase-3 and mouse-anti-caspase-8 from Cell Signalling Technology, mouse-anti-caspase-3 from Transduction Laboratories (Lexington, KY), rabbit-anti-poly-ADP-ribose polymerase (PARP) from Roche (Mannheim, Germany) and mouse-anti-actin from ICN Biomedicals (Zoetermeer, The Netherlands). Western blot analyses were performed in triplicate. The Bradford assay was used to determine protein concentrations. Samples containing 20 µg lysate were used. Membranes were stained with Ponceau S to check for equal protein loading. Actin expression levels served as loading control.

Caspase enzyme activity after apoptosis induction by rhTRAIL

Cells were incubated with rhTRAIL 0.1 µg/ml for 5 hours at 37°C. Activity of caspase-3 and caspase-8 were assayed according to the manufacturer's instructions using the caspase-specific fluorescence peptide substrates Ac-DEVD-AFC and Ac-IETD-AFC (Biomol Tebu-bio, Heerhugowaard, the Netherlands) respectively. Relative caspase activity was obtained by comparing the treated to the untreated samples. All experiments were performed in triplicate.

Patients

All patients above 40 years of age, without a history of colitis, HNPCC or FAP undergoing colonoscopy at the University Medical Center Groningen during 2004 were approached for the adenoma study. All patients above 18 years of age undergoing colonoscopy in January and February 2005, without a history of colitis, colorectal cancer, HNPCC, FAP or coagulation disorders and not taking medication influencing coagulation were approached for the normal colon biopsy study. All patients were informed about the study and gave written informed consent. The study protocol was approved by the medical ethical review committee of the University Medical Center Groningen.

Clinical specimens: colorectal polyps and normal colon epithelium

Colorectal polyps, 0.5-2 cm in diameter were put in medium (RPMI supplemented with 10% foetal calf's serum) at room temperature directly after endoscopic resection and processed immediately. A section of the polyp, up to 100 mm³ depending on the size of the polyp, was removed by the pathologist and was transported to the laboratory in medium. The remaining section of the polyp was prepared for routine histology. Three biopsies of normal colon mucosa were obtained from the sigmoid of patients without macroscopic abnormalities at colonoscopy. One biopsy of normal colorectal mucosa was prepared for routine histology and the other two were transported directly to the laboratory in medium. In the laboratory, the removed section of polyp (n=48) was cut into two equal pieces. One piece of polyp was incubated in tissue culture medium (as described by Tong *et al* ¹⁵) and the other piece was incubated in tissue culture medium with rhTRAIL 1 µg/ml. In five cases, the polyp tissue segment was large enough to be divided into four pieces to determine whether apoptosis in freshly removed adenomas was caspase dependent. The first two pieces were incubated as described above, the third in tissue culture medium with 50 µM of the caspase inhibitor zVAD-fmk and the fourth in tissue culture medium with a combination of rhTRAIL and zVAD-fmk. All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 5 hours. The incubation time was chosen based on preliminary observations indicating that apoptosis induction by rhTRAIL in freshly removed polyps already occurs after 3 hours, whereas, after long incubation periods the quality of the material decreases gradually in time, making evaluation more difficult. The biopsies of normal colon mucosa were also incubated for 5 hours in tissue culture medium, one biopsy with, and one without rhTRAIL 1 µg/ml. After incubation all polyp pieces and biopsies were fixed in formalin and embedded in paraffin.

Histological classification

Histological classification of polyps and normal colorectal mucosa was carried out in H&E stained slides by the pathologist (JW). Adenomas and degree of dysplasia (low or high) were defined according to the World Health Organization guidelines ¹⁶. In adenomas with high-grade dysplasia the percentage of the adenoma in which high-grade dysplasia was present was estimated semi-quantitatively (< 25%, 25-50%, 50-75%). The location of the adenomas was retrieved from endoscopy or pathology reports. The biopsies of normal colorectal mucosa were examined for signs of inflammation or dysplasia and, if present, the biopsies from that patient were excluded from further analysis.

Determination of apoptosis in clinical specimens

All paraffin embedded clinical specimens were H&E stained, to determine the morphological characteristics and the degree of apoptosis, and were stained for cleaved caspase-3 (Cell Signalling Technology) following manufacturers instructions to determine the degree of apoptosis. Two investigators (WB, MJ) blindly evaluated the slides. If there was no agreement initially, slides were re-evaluated under a multi-headed microscope. The size of the cultured adenoma pieces was measured as the maximal diameter and the area of the tissue section in which intact crypts were visible was estimated semi-quantitatively (0-50%, 50-100%). Adenomas of which the control sections contained less than 50% evaluable crypts were considered to be too damaged for further analysis. At a 40x magnification each tissue section was divided into three to eight identical areas of approximately 1 mm², depending on the size of the tissue section, and at least one intact crypt from each area was chosen at random. At 400x magnification cells were counted in these predefined crypts. In all cases at least 1,000 epithelial cells were counted. In H&E stained slides apoptotic cells were identified using morphological characteristics including cell shrinkage, nuclear condensation and formation of apoptotic bodies. Cleaved caspase-3 positivity was identified as brown cytoplasmic staining. The degree of apoptosis was expressed as the percentage of apoptotic cells of the total number of cells counted. Percentage of specific TRAIL-induced apoptosis was calculated, from the results of the H&E stained slides, as follows: $100\% \times [(experimental\ apoptosis\ (\%) - spontaneous\ apoptosis\ (\%)) / (100\% - spontaneous\ apoptosis\ (\%))]^{17}$.

Statistical analysis

SPSS for Windows software (SPSS Inc., Chicago, IL) was used in all statistical analyses. For comparisons between treated and untreated tissue sections the paired Wilcoxon signed rank test was used. The Spearman rank correlation test was used to determine the relationship between two variables. p values of < 0.05 were considered significant.

Results

RhTRAIL induces apoptosis in adenoma cell lines

The adenoma cell lines both expressed the receptors, DR4 and DR5 (figure 1a). RhTRAIL induced apoptosis in both cell lines. As shown in figure 1b, incubation with rhTRAIL for 5 hours induced apoptosis in both the VACO 235

and the VACO 330 cells. Apoptosis induction by rhTRAIL could be inhibited using a pan-caspase inhibitor (figure 1b). The amount of apoptosis was similar in the adenoma cell lines and in the intermediately TRAIL-sensitive colon cancer cell line LOVO (figure 1b). Western blot analysis showed that induction of apoptosis was accompanied by activation of caspase-8 and caspase-3 and cleavage of the early apoptosis marker PARP (Figure 1c). Caspase enzyme activity assays also showed activation of caspase-8 and caspase-3 by rhTRAIL (Figure 1d). In summary, rhTRAIL induces caspase dependent apoptosis in human colon adenoma cell lines.

RhTRAIL induces apoptosis in adenomas, but not in normal colon epithelium

To elucidate whether (subgroups of) freshly removed adenomas are sensitive to rhTRAIL-induced apoptosis a series of polyps (n = 48) was tested. Five polyps were excluded from further analysis since they were classified as hyperplastic polyps or juvenile polyps and five others were excluded because less than 50% of the crypts were evaluable.38 adenomas were included in the final analysis. Patient characteristics are shown in table 1.

Table 1: Patient characteristics.

	Adenomas	Normal Colon
N	38	15
Male (%)	61	47
Mean age in years (range)	65 (41-87)	51 (24-74)
Localization (%)		
- Ascending / transverse	16	-
- Descending / sigmoid	68	100
- Rectum	16	-
Low-grade dysplasia (%)	45	-
High-grade dysplasia (%)	55	-

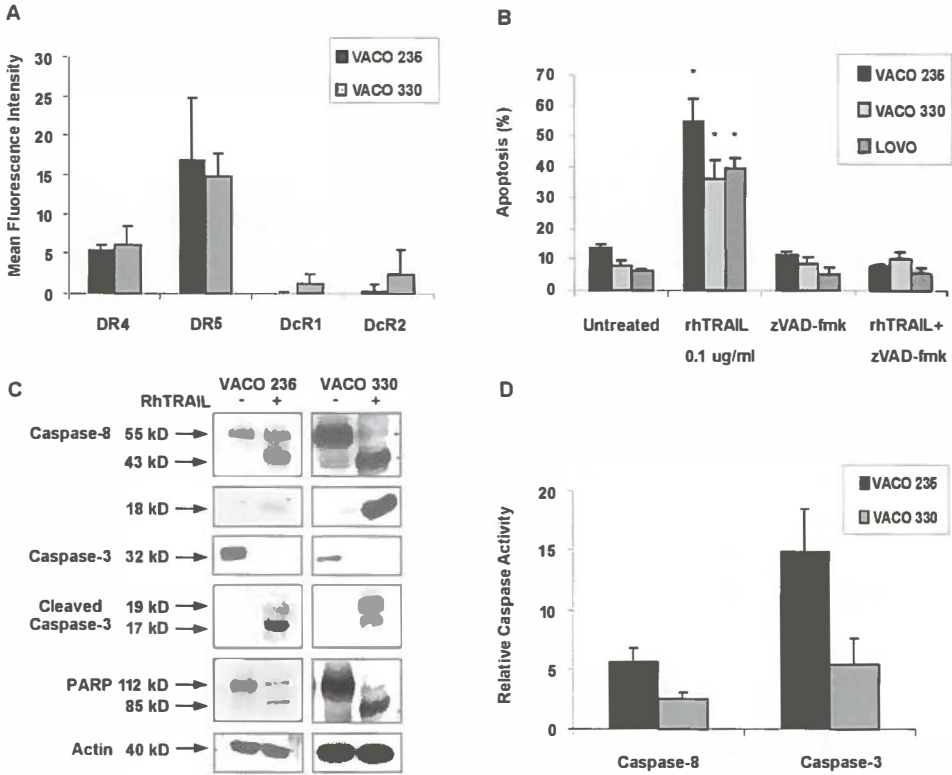


Figure 1: (A) Membrane expression of the TRAIL receptors DR4, DR5, DcR1 and DcR2 in the adenoma cell lines VACO 235 and VACO 330 as determined by flow cytometry. Values are expressed as the mean fluorescence intensity and are mean \pm SEM of at least three independent experiments. (B) Degree of apoptosis (%) in VACO 235, VACO 330 and LOVO after treatment with rhTRAIL and/or zVAD-fmk for 5 hours as determined by immunocytochemistry for cleaved caspase-3. Experiments are performed at least three times, values are means \pm SEM. *: The percentage of apoptotic cells is higher in the rhTRAIL treated group as compared to the other three treatments for each cell line ($p < 0.05$). (C) Western blot analysis of cleavage of caspase-8, caspase-3 and PARP in adenoma cell lines. RhTRAIL induces cleavage of caspase-8 into the intermediate 41/43 kD product and the active p18 sub-unit. If cells are apoptotic, caspase-3 (32 kD) is cleaved, resulting in two fragments (17 and 19 kD) and full-length PARP (112 kD) is cleaved, resulting in an 85 kD piece. One representative of at least three different experiments is shown. Actin (40 kD) is shown as loading control. (D) Caspase activity in adenoma cell lines after 5 hours incubation with rhTRAIL 0.1 μ g/ml. Caspase-3 and -8 activity was determined using fluorimetric assays. Caspase activity is expressed as the ratio of treated to untreated cells. Experiments are performed at least three times, values are means \pm SEM.

Figure 2 shows two pieces of the same adenoma with high-grade dysplasia, one treated with rhTRAIL showing extensive apoptosis (b) and one untreated in which normal cell morphology is visible (a).

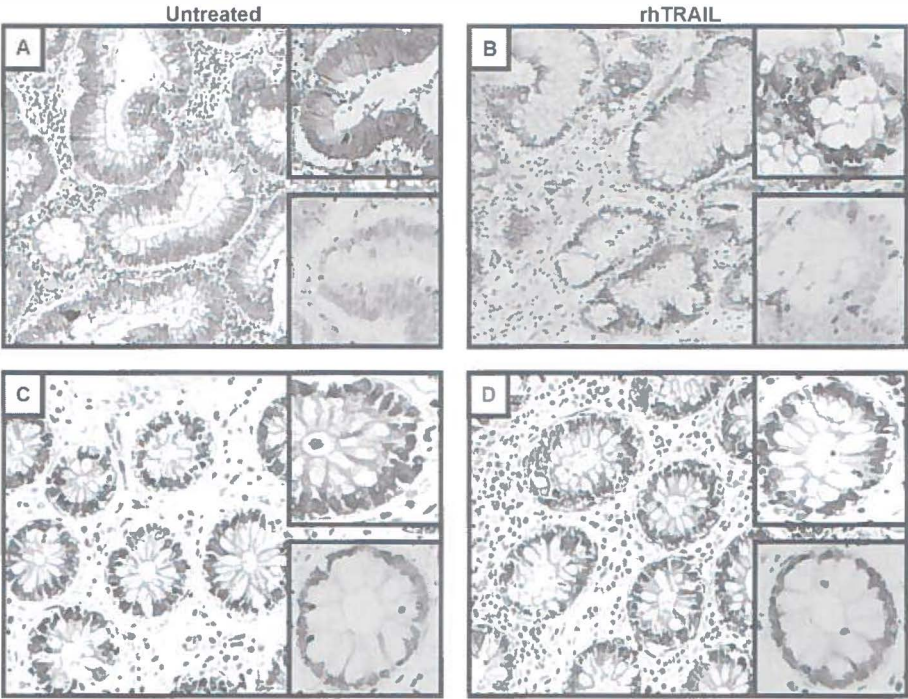


Figure 2: Colon tissue sections after 5 hours of incubation. Main figures: H&E stained (original magnification 200x). Upper inserts: H&E stained (original magnification 400X). Lower inserts: stained for cleaved caspase 3 (original magnification 400X). Adenoma with high-grade dysplasia: (A) untreated and (B) treated with 1 µg/ml rhTRAIL. Normal colon epithelium: (C) untreated and (D) treated with 1 µg/ml rhTRAIL... See appendix 3, page 177 for colour figure.

In order to show that this apoptosis was caspase dependent and not an artefact of the culture model, we incubated five of the larger adenomas in combinations of the broad-spectrum caspase inhibitor zVAD-fmk and rhTRAIL. RhTRAIL also induced apoptosis in these adenomas, as determined in H&E stained slides and slides stained for cleaved caspase-3, and this apoptosis could be completely inhibited by zVAD-fmk (figure 3). This proves that the

rhTRAIL-induced apoptosis in adenomas is mediated through caspase activation. There was no difference in degree of apoptosis between untreated and zVAD-fmk treated adenoma tissue pieces indicating that zVAD-fmk alone did not cause or prevent apoptosis. In the large series of adenomas tested, no difference was observed in the mean size of the tissue pieces between the rhTRAIL treated and the untreated groups ($3.4 \text{ mm} \pm 0.2 \text{ mm}$ vs. $3.4 \text{ mm} \pm 0.2 \text{ mm}$, mean \pm SEM). Adenoma tissue segments treated with rhTRAIL had a higher mean degree of apoptosis than untreated segments (table 2). Subgroup analysis revealed that apoptosis induction was higher in the rhTRAIL treated segments than in the paired untreated segments in adenomas with high-grade dysplasia ($n=21$), but not in adenomas with low-grade dysplasia ($n=17$) (table 2, figure 4a/b).

In normal sigmoid epithelium ($n=15$) the mean degree of apoptosis in untreated segments was lower than in the untreated adenoma segments. There was no difference in the mean degree of apoptosis between treated and untreated biopsies of normal colonic mucosa (table 2, figure 2c/d).

Table 2: Degree of apoptosis (%) in tissue pieces and biopsies cultured in tissue culture medium (untreated) or in tissue culture medium with rhTRAIL $1 \text{ } \mu\text{g/ml}$.

Group	N	H&E			Cleaved caspase-3		
		Untreated mean \pm SEM	RhTRAIL mean \pm SEM	p value ¹	Untreated mean \pm SEM	RhTRAIL mean \pm SEM	p value ¹
All adenomas	38	17 ± 2	26 ± 3	0.003	11 ± 2	16 ± 3	0.030
High-grade dysplasia	21	17 ± 2	34 ± 5	0.002	14 ± 3	21 ± 4	0.048
Low-grade dysplasia	17	16 ± 3	17 ± 3	NS*	7 ± 2	9 ± 2	NS*
Normal colon	15	6 ± 1	6 ± 1	NS*	5 ± 1	6 ± 1	NS*

¹paired Wilcoxon signed rank test, *not significant

In general, larger tissue pieces were easier to handle than smaller tissue pieces resulting in less mechanical damage. Concomitantly, larger untreated

tissue pieces had lower degrees of apoptosis ($R = -0.317$, $p = 0.05$). To adjust for these variations in apoptosis in the control tissue segments, the degree of treatment specific apoptosis was also calculated. Using this calculation, adenomas with high-grade dysplasia were again shown to be sensitive to rhTRAIL induced apoptosis, while adenomas with low-grade dysplasia and normal colon epithelium were not sensitive. Furthermore, adenomas with a higher percentage of high-grade dysplasia were shown to be more sensitive to rhTRAIL-induced apoptosis ($R = 0.6$, $p = 0.004$, figure 4c/d).

Discussion

The pro-apoptotic TRAIL receptors DR4 and DR5 are immunohistochemically expressed in colorectal adenomas. However, immunohistochemical studies cannot predict whether these receptors induce apoptosis following the binding of rhTRAIL. In the present study, we show that two human adenoma cell lines, one derived from a villous adenoma and the other from a tubular adenoma, express DR4 and DR5 and are both sensitive to rhTRAIL induced apoptosis. In a clinically more relevant model, freshly removed human colorectal adenomas with high-grade dysplasia, but not those with low-grade dysplasia, were also sensitive to rhTRAIL-induced apoptosis. Importantly, freshly removed normal human colonic epithelium was not sensitive to rhTRAIL.

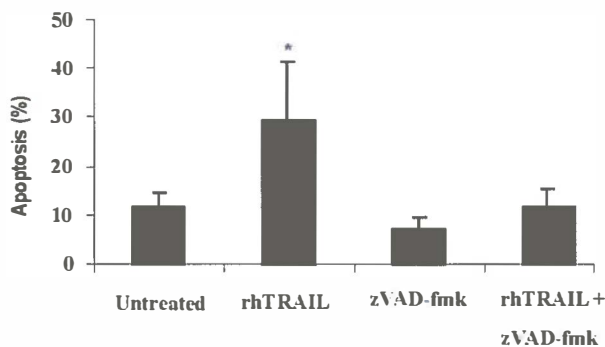


Figure 3: Degree of apoptosis in adenoma tissue pieces as determined by morphological characteristics in H&E stained slides: untreated and treated with 1 $\mu\text{g/ml}$ rhTRAIL, 50 μM zVAD-fmk or rhTRAIL and zVAD-fmk. Values are means \pm SEM for 5 patients. *: The percentage of apoptotic cells is higher in the rhTRAIL treated adenoma pieces as compared to the other three treatments for each adenoma ($p < 0.05$).

It is well known that in contrast to normal cells, cancer cell lines and also immortalized and transformed normal cell lines are sensitive to rhTRAIL-induced apoptosis. This is not only true for artificially immortalized cells (viral and telomerase immortalization) but also for spontaneously transformed cells¹⁸⁻²². This had not previously been reported for colorectal adenoma cells which can, under certain conditions, be grown in culture and can therefore be described as transformed cells. The rhTRAIL sensitivity of the two adenoma cell lines used in this study is, therefore, in line with previous results in transformed cell lines. In contrast, Hague *et al* reported that four adenoma cell lines which were similar to those used in this study, were not sensitive to 1 µg/ml TRAIL, unless transformed to a malignant phenotype *in vitro*²³⁻²⁶. The histidine-tagged TRAIL used by Hague *et al* is, however, known to be less potent than native rhTRAIL at inducing cell death in a number of cancer cell lines^{27,28}.

Our cell line results indicate that adenomas could be sensitive to rhTRAIL induced apoptosis. However, the number of adenoma cell lines available is limited and their validity as a model for adenomas is often questioned. To determine whether human colorectal adenomas are truly sensitive to rhTRAIL it is necessary to use a model that is closer to the *in vivo* situation, such as a short-term explant culture model. In the present study we show that, in this clinically more relevant model, freshly removed human colorectal adenomas with high-grade dysplasia, but not those with low-grade dysplasia, are sensitive to rhTRAIL induced apoptosis. Importantly, in the same model, freshly removed normal human colon epithelium is not sensitive. The latter is in agreement with toxicity studies in rodents, monkeys and chimpanzees and a study in isolated human normal colon crypts which also demonstrated that native soluble rhTRAIL is not toxic for normal cells²⁸⁻³¹. The advantage of using short-term culture of adenoma explants as opposed to single-cell suspensions and adenoma cell lines is that they contain representative, unselected combinations of cell populations in an environment with stroma and intact cell-cell interactions³²⁻³⁴. The amount of apoptosis in the cultured untreated control segments was relatively high as compared to untreated samples from the same patient which were directly fixed in formalin (data not shown) and also in comparison to the cultured normal colonic epithelium. This apoptosis is probably caused by detachment from surrounding connective tissue on the one hand and damage occurring between resection and incubation on the other, the latter being more prominent for the adenoma sections than for the biopsies of normal colonic epithelium³⁵. This is supported by the fact that this apoptosis was not inhibited by a pan-caspase inhibitor and was higher in the more difficult

to handle smaller adenoma segments. The rhTRAIL sensitivity of high-grade dysplastic adenomas in this culture model is in line with the results in the cell line model suggesting that the rhTRAIL sensitivity is not an artifact of the culture model. In a small subset of cases apoptosis was higher in the control segment compared to the treated segment.

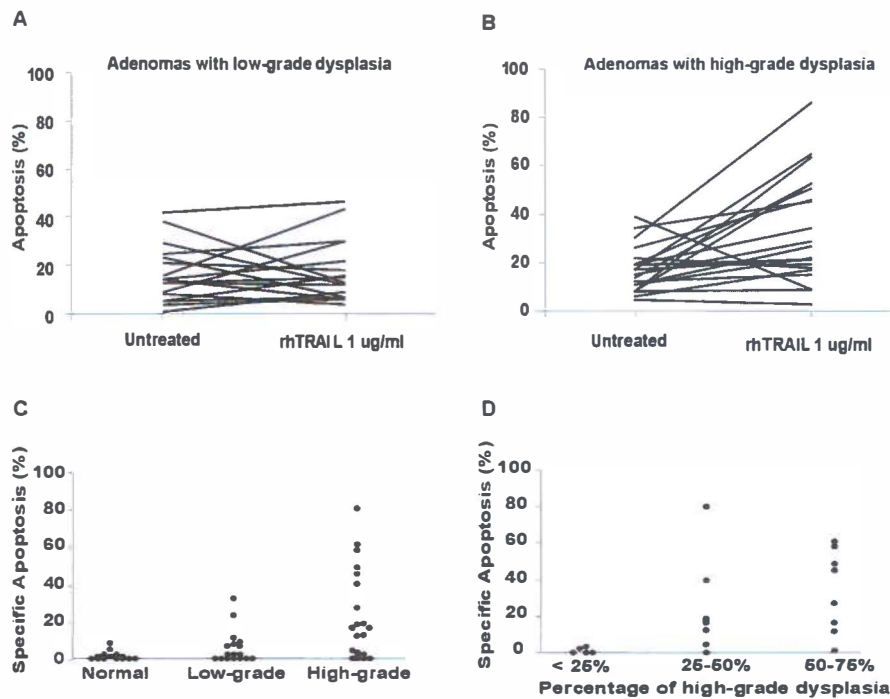


Figure 4: The degree of apoptosis in paired untreated and treated tissue samples (the control and treated sample of each pair joined by a line) as determined by morphological characteristics in H&E stained slides. Adenomas with low-grade (A) and high-grade (B) dysplasia. (C) Percentage of specific apoptosis (%) in normal colorectal mucosa and adenomas with low- and high-grade dysplasia as calculated for paired untreated and treated tissue pieces using morphological characteristics in H&E stained slides. (D) Percentage of specific apoptosis (%) in adenomas with high-grade dysplasia with < 25%, 25-50% and 50-75% of the adenoma showing high-grade dysplasia. Percentage of specific apoptosis was calculated as follows: $100\% \times [(experimental\ apoptosis\ (\%) - spontaneous\ apoptosis\ (\%)) / (100\% - spontaneous\ apoptosis\ (\%))]$.

This is also most likely to be a consequence of the culture model as described above. Another possibility is that rhTRAIL induced inhibition of apoptosis in

these adenomas. rhTRAIL inhibition of apoptosis of cancer cell lines has been described, however, this is rare and mostly a consequence of mutations rarely found in colorectal adenomas or carcinomas^{36,37}.

Subgroup analysis showed that only adenomas with high-grade dysplasia were sensitive to rhTRAIL-induced apoptosis. Furthermore, adenomas with high-grade dysplasia were more sensitive when a larger percentage of the adenoma was highly dysplastic. These results indicate that TRAIL sensitivity is acquired relatively late in the adenoma-to-carcinoma sequence³⁸. Immunohistochemical expression of the TRAIL receptors is the same in adenomas with low- and high-grade dysplasia, therefore this cannot explain the observed difference in sensitivity⁸. Inactivation of the TRAIL receptors by mutation or methylation is thought to be rare in colorectal cancer³⁷. It is, however, possible that the availability of the receptors at the membrane is different between high- and low-grade dysplastic adenomas. Furthermore, little is known about downstream expression levels of genes involved in the TRAIL apoptosis pathway in adenomas and whether these differ between adenomas with low- and high-grade dysplasia. It has been shown that mutations in the oncogene Ras, which are acquired during the adenoma-carcinoma sequence, can sensitize cells to rhTRAIL-induced apoptosis and this mechanism could be, at least in part, responsible for the sensitivity of adenomas with high-grade dysplasia^{39,40}.

Early clinical trials involving rhTRAIL and agonistic TRAIL receptor antibodies are currently in progress in cancer patients (<http://www.hgsi.com>). The rhTRAIL-sensitivity of a premalignant lesion such as a colorectal adenoma is interesting and indicates that the application of rhTRAIL, agonistic TRAIL receptor antibodies or other, yet to be developed, TRAIL-receptor agonists may be possible in this setting. A potential application could be the downsizing of endoscopically irresectable adenomas with high-grade dysplasia to allow endoscopic removal. In patients with HNPCC, this could prevent a subtotal colectomy and in patients with FAP with ileorectal anastomosis it might mean that proctectomy can be averted. It is interesting to note that non-steroidal anti-inflammatory drugs (NSAIDs), which are frequently used in the treatment of these latter patients, have been shown to potentiate TRAIL-induced apoptosis in colorectal cancer cells *in vitro*³⁷.

In conclusion, rhTRAIL induces apoptosis in colorectal adenoma cell lines and also in freshly removed human colorectal adenomas with high-grade dysplasia, but not in those with low-grade dysplasia or in normal colon epithelium. TRAIL

based therapy could therefore be a potential adjuvant in the treatment of adenomas with high-grade dysplasia.

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Chapter 4

Regulation of TRAIL receptor expression by β -catenin in colorectal tumours

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Abstract

TNF-related apoptosis-inducing ligand (TRAIL) is a potential novel anti-cancer drug and the expression of its pro-apoptotic receptors, DR4 and DR5, increases during the colorectal adenoma-carcinoma sequence. The aim of this study was to investigate whether β -catenin is involved in the regulation of these receptors. In human colorectal adenoma and carcinoma cell lines, down-regulation of β -catenin resulted in lower total DR4 and DR5 protein levels. Similarly, cell membrane expression of DR4 and DR5 was reduced after down-regulation of β -catenin in colon cancer cell lines and, furthermore, β -catenin suppression decreased rhTRAIL sensitivity. β -catenin-independent down-regulation of TCF-4 signalling, however, did not affect TRAIL receptor expression or rhTRAIL sensitivity. Colorectal tumours with aberrant (cytoplasmic and nuclear) β -catenin expression had a higher percentage of immunohistochemical DR4 and DR5 staining per tumour than those with membranous β -catenin staining. Furthermore, aberrant β -catenin staining co-localized with both DR4 and DR5 expression in 92 % of adenomas. In human colorectal carcinomas aberrant β -catenin expression was present in most cases and DR4/5 expression was largely homogenous. In conclusion, the gradual increase in TRAIL-receptor expression during colorectal carcinogenesis is at least partially mediated through increased β -catenin expression.

Introduction

Tumour-necrosis-factor related apoptosis inducing ligand (TRAIL) is a cytokine which can induce apoptosis by binding to its pro-apoptotic receptors DR4 and/or DR5¹. The immunohistochemical expression of these receptors is stronger in colorectal adenomas and carcinomas in comparison with normal colon epithelium in both sporadic and hereditary cases². Furthermore, recombinant human (rh) TRAIL can induce apoptosis in human colon cancer cell lines, human colon adenoma cell lines and human colorectal adenomas^{3,4}. In view of this, and its minimal toxicity for normal cells *in vitro* and *in vivo*, rhTRAIL is an exciting potential drug for the treatment and prevention of colorectal cancer⁵. The increase in TRAIL receptor expression during colorectal carcinogenesis is so far unexplained but could be related to activation of the Wnt pathway.

The initiation and progression of the majority of colorectal carcinomas involves activation of the Wnt pathway⁶. During colorectal carcinogenesis, the Wnt-pathway is activated due to APC or β -catenin mutations leading to inadequate degradation of β -catenin in the cytoplasm of the cells. This results in nuclear accumulation of β -catenin and transcriptional activation of T-cell factors (TCFs), which regulate genes involved in processes such as cell proliferation and apoptosis⁶. The accumulation of β -catenin in both cytoplasm and nucleus, as well as the over-expression of TCF-4-target gene products, is seen in colorectal adenomas and to a greater extent in colorectal cancers^{7,8}.

The hypothesis of the present study is that β -catenin accumulation leads to the upregulation of the TRAIL receptors DR4 and DR5. We therefore investigated whether increased expression of β -catenin and subsequent TCF-4 activation are involved in the increase of DR4 and DR5 expression during colorectal carcinogenesis. Firstly human colon adenoma and carcinoma cell lines in which β -catenin and/or TCF-4-signaling could be down-regulated were investigated with regard to TRAIL receptor expression and rhTRAIL sensitivity. To further investigate the hypothesis patient material was investigated by analyzing immunohistochemical staining of DR4 and DR5 in relation to the expression of β -catenin in human colorectal tumours.

Materials and methods

Cell lines

Ls174 colon cancer cell lines (LS174T-BcatRNAi, Ls174T-dnTCF-4 and Ls174T) containing doxycyclin-inducible expression plasmids were used⁹. The “LS174T-BcatRNAi” has an inducible β -catenin-short-hairpin (sh)RNA vector which allows the rapid production of siRNAs on doxycyclin induction. The “Ls174T-dnTCF-4” has a doxycyclin-inducible plasmid encoding N-terminally truncated versions of TCF-4. These dominant-negative TCF-4 (dnTCF-4) proteins do not bind β -catenin and act as potent inhibitors of TCF-4 signalling. The “Ls174T” is stably transfected with the doxycyclin responsive promotor and an empty vector as a control¹⁰. Using this cell line model it is possible to down-regulate Wnt pathway activity at the β -catenin or the TCF-4 level. The cell lines were cultured in RPMI enriched with 5% foetal calf’s serum (FCS) at 37 °C in a humidified atmosphere with 5% CO₂. The cells were harvested by treatment with trypsin for 5-10 min at 37 °C. The cells were put under selection with blasticidin 10 μ g/ml and zeocin 500 μ g/ml for one week every month. To induce dnTCF-4 expression and β -catenin RNAi production, doxycyclin was added to the medium at a final concentration of 1 μ g/ml. The adenoma cell lines VACO-235 and VACO-330 are human adenoma cell lines, derived from a villous and tubular adenoma respectively, which exhibit features of well differentiated epithelial cells, including cell polarity, microvilli and junctional complexes¹¹. The adenoma cell lines were cultured as described previously¹¹.

β -catenin RNA interference in adenoma cell lines

SiRNAs specific for human β -catenin were synthesized by Eurogentec (Seraing, Belgium): 5'-GUG GGU GGU AUA GAG GCU C99-3' (sense) and 5'-GAG CCU CTA TAC CAC CCA C99-3' (antisense). Double-stranded RNA molecules specific for the luciferase (Luc) gene served as a control. The sequences for Luc RNA molecules were 5'-CUU ACG CUG AGU ACU UCG AdTdT-30 (sense) and 5'-UCG AAG UAC UCA GCG UAA GdTdT-30 (antisense)³. Flow cytometric analysis of VACO 235 and VACO 330 cells transfected with fluorescein-5-isothiocyanate-labeled oligonucleotides revealed a transfection efficiency of 85% and 65% respectively. VACO-235 and VACO-330 cells were transfected in six-well plates with 10 ml of 20 μ M siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). After 48 h, cells were harvested for protein isolation or FACS-analysis.

Analysis of membrane expression of TRAIL receptors using flow cytometry

The Ls174 transfectant cell lines were induced with doxycyclin for 48 h prior to analysis. VACO-235 and VACO-330 cells were transfected with siRNA for β -catenin for 48 h, as described above. Cells were harvested as described previously ¹¹ and resuspended in flow cytometry medium (phosphate-buffered saline, 2% FCS, 1% sodium azide). Analysis of TRAIL-receptor membrane expression was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL) and cells were stained as described earlier ³. The following antibodies were used: huTRAILR1-M271 for DR4 and huTRAILR2-M413 for DR5 (a gift from Amgen, Seattle, WA). Membrane receptor expression is shown as mean fluorescence intensity of all analyzed cells. All experiments were performed in triplicate.

Western blot analysis

The Ls174 transfectant cells were induced with doxycyclin for 48 h or treated with cycloheximide 2 μ g/ml (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 24 h prior to analysis. Cells were harvested as described above. Western blot analysis was carried out as described previously ¹². The following primary antibodies were used: a mouse monoclonal specific for β -catenin (1:2000, Transduction Laboratories), a goat polyclonal IgG specific for DR4 (1:500; clone C-20, Santa Cruz Biotechnology), a rabbit polyclonal IgG specific for DR5 (1:500; Oncogene Research), a rabbit polyclonal specific for c-myc (1:500, clone n-262, Santa Cruz), a mouse monoclonal specific for FLAG (1:1000, Sigma-Aldrich) and a mouse monoclonal specific for actin (1:10,000, ICN Biomedicals, Zoetermeer, The Netherlands). The secondary antibodies were labelled with horse radish peroxidase (all from DAKO, Heverlee, Belgium) and chemiluminescence was detected using the ECL-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Almere, The Netherlands). Western blot analyses were performed in triplicate. The Bradford assay was used to determine protein concentrations in all samples. In all experiments, samples containing 20 μ g lysate were used and all membranes were stained with Ponceau S to check for equal protein loading. Actin expression levels were used as a loading control.

Apoptosis induction by rhTRAIL

For the Ls174 transfectant cell lines 10,000 cells were seeded in 96-well plates, were induced using doxycyclin and were incubated at 37 °C for 48 h. rhTRAIL (0.1 or 1.0 μ g/ml) was added for the last 5 h of incubation. RhTRAIL was

produced non-commercially in co-operation with IQ-Corporation (Groningen, the Netherlands) as described previously ¹³. Apoptosis was identified by staining nuclear chromatin with acridine orange (10 µm/ml) and identifying the appearance of apoptotic bodies and/or chromatin condensation by fluorescence microscopy. Apoptosis was expressed as percentage apoptotic cells in a culture. Apoptosis experiments were performed at least three times.

Immunohistochemistry for β -catenin, DR4 and DR5 in human adenomas and carcinomas

Immunohistochemistry for β -catenin, DR4 and DR5, was carried out in patient derived colorectal adenomas and carcinomas. Paraffin-embedded colorectal adenomas and carcinomas were retrieved from the Department of Pathology of the University Medical Center Groningen. The origin and characteristics of this group of tumours, as well as the expression of DR4 and DR5 have been described previously ¹⁴. A number of tumours from the original report were excluded in the present study since insufficient material was available for further analysis. Immunohistochemical staining was carried out as described previously ¹⁴. The variations in staining intensity of DR4/5 positive cells were very small, therefore immunohistochemical DR4/5 expression was scored as either positive or negative for each individual cell. DR4 and DR5 expression for each tumour was estimated semi-quantitatively as the percentage of positive cells within the tumour, where tumours with less than 10% positive cells were considered negative.

For β -catenin, antigen retrieval was performed by microwave treatment of the slides for 8 min at 700 W in 0.01 M citrate buffer (pH 6.0). A mouse monoclonal antibody for β -catenin (1:1000; Transduction Laboratories, Lexington, KY) was applied for 60 min. This was followed by incubation with appropriate secondary and tertiary antibodies and peroxidase activity was visualized with diaminobenzidine. As a positive control, colon carcinoma and normal colon epithelium were used. In colon cancer, β -catenin staining is localized in the cytoplasm and nucleus while in normal colon epithelium β -catenin staining is membranous. The pattern of staining for β -catenin was recorded as membranous, cytoplasmic or nuclear and the percentage of cells with nuclear or cytoplasmic staining was estimated. β -catenin staining was defined as nuclear when at least 10% of the epithelial cells had nuclear staining.

To further investigate the relationship between β -catenin and DR4/DR5 staining in colorectal adenomas, co-localization of staining results was investigated in

serial slides of the 13 smallest adenomas. Larger adenomas would have to be photographed in multiple sections making analysis more difficult. To determine co-localization, each adenoma was photographed at a 25x magnification. Image-pro plus, version 4.6 (MediaCybernetics, Silver Spring, MD) was used to synchronize the orientation of the adenomas and to place a grid over the adenomas. Using this grid each adenoma was divided into equal squares, the number (mean: 180, range: 84-490) depending on the size of the adenoma. For DR4 and DR5 staining each square containing epithelial cells was evaluated as positive or negative. For β -catenin staining, squares with cytoplasmic and/or nuclear staining were considered positive and squares with membranous staining were considered negative.

Statistical analysis

SPSS for Windows software (SPSS Inc., Chicago, IL) was used in all statistical analyses. P values of < 0.05 were considered significant. Comparisons between different groups regarding histopathological characteristics and expression of proteins were evaluated using the Mann Whitney-U test for two independent samples which are not normally distributed and linear regression analysis. To determine co-localization between two stainings the kappa coefficient was determined. The McNemar test was used to investigate discordance between the staining results.

Results

Down-regulation of β -catenin leads to reduced DR4 and DR5 expression and reduced TRAIL sensitivity

The effect of down-regulation of β -catenin on DR4 and DR5 expression was investigated in human colon carcinoma and adenoma cell line models. All cell lines used exhibit basic membranous expression of DR4 and DR5 (data not shown). Down-regulation of β -catenin in the human LS174T-Bcat-RNAi carcinoma cell line following doxycyclin-induced expression of β -catenin shRNA led to a time-dependent reduction in the protein expression of the Wnt-target gene c-myc (figure 1a). In the adenoma cell lines, β -catenin down-regulation, using a β -catenin-siRNA approach, also resulted in a decreased c-myc expression (figure 1b). This demonstrates functional inhibition of the Wnt-pathway, in both carcinoma and adenoma cell line models. Down-regulation of β -catenin was accompanied by reduced protein expression of both DR4 and DR5 in all cases (figure 1a,b). These reductions in DR4 and DR5 expression

were comparable to those achieved by treating the cells with the protein synthesis inhibitor cycloheximide for 24 h (data not shown).

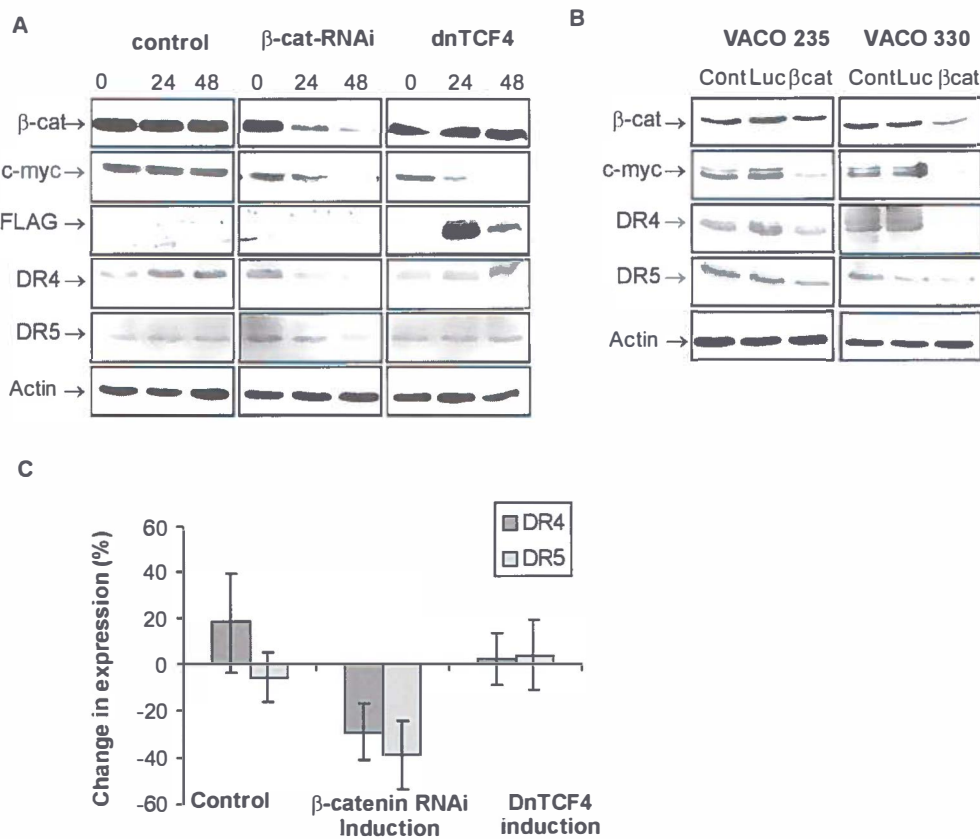


Figure 1: LS174T, LS174T-Bcat-RNAi and the LS174T-dnTCF-4 cells were treated with doxycyclin for 0, 24 and 48 h to induce expression of target sequences. (A) Western blot analysis of β -catenin, c-myc, FLAG, DR4 and DR5 expression in LS174T cells. One representative of at least three different experiments is shown. Actin is shown as loading control. VACO 235 and VACO 330 cells were exposed to oligofectamine alone (cont) or transfected with siRNA duplexes directed against luciferase (luc) as control siRNA or against β -catenin (β cat) for 48h. (B) Western blot analysis of β -catenin, c-myc, DR4 and DR5 expression in VACO 235 and VACO 330 cell lines. One representative of at least three different experiments is shown. Actin is shown as loading control. (C) Membrane expression of the TRAIL receptors DR4 and DR5 in the LS174T, LS174T-Bcat-RNAi and the LS174T-dnTCF-4 as determined by flow cytometry. Values are expressed as the reduction in the mean fluorescence intensity after 48 h induction with doxycyclin and are mean \pm SD of at least three independent experiments.

Membrane expression of DR4 and DR5 was also reduced after down-regulation of β -catenin in the colon carcinoma cell line model (29 % and 39 % reductions, respectively (figure 1c)).

The LS174T colon cancer cell lines were sensitive to rhTRAIL induced apoptosis (38 % apoptosis after 5h of rhTRAIL). Down-regulation of β -catenin in this cell line model reduced rhTRAIL induced apoptosis to 14 % apoptosis after 5 h of rhTRAIL (figure 2). This indicates that the reduction in DR4/5 expression is associated with a reduced sensitivity to rhTRAIL. All reported effects were not seen in the control cell line, LS174T, indicating that it is not an effect induced by the addition of doxycyclin itself. β -catenin can bind to the transcription factor TCF-4 in the nucleus and so activate transcription of TCF-4-target genes. The LS174T-dnTCF-4 cell line was used to investigate whether the effect of β -catenin levels on DR4/5 expression is TCF-4-mediated.

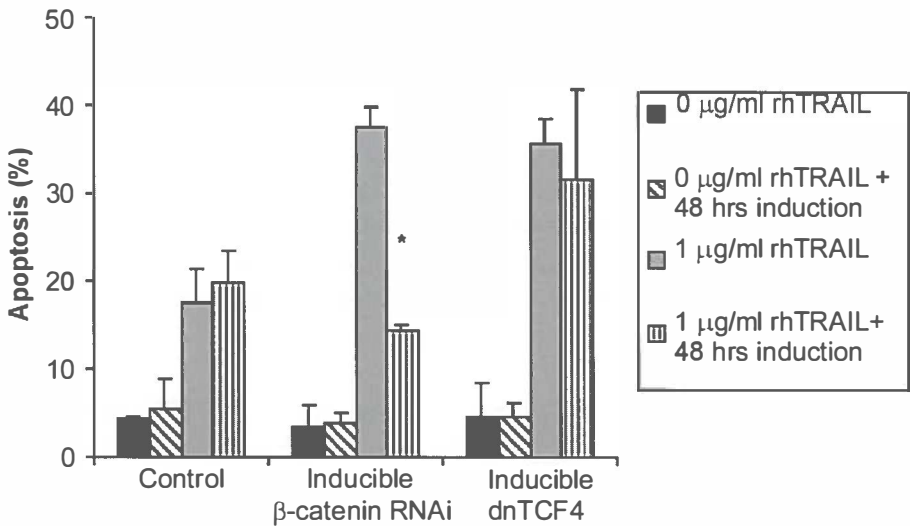


Figure 2: LS174T, LS174T-Bcat-RNAi and the LS174T-dnTCF-4 control and rhTRAIL 1 μ g/ml, with and without 48 h of induction. Apoptosis was determined by acridine orange staining and is expressed as a percentage of the total number of cells counted. *The percentage of apoptosis in LS174T-Bcat-RNAi cells was lower after induction of the RNAi expression constructs ($p < 0.05$).

Doxycyclin-induced expression of FLAG-tagged dnTCF-4 protein was shown and the dominant-negative effect of dnTCF-4 expression was demonstrated by the reduced protein expression of the TCF-4-target gene c-myc (figure 1a). Expression of dnTCF-4 did not influence protein or membrane levels of DR4 or DR5 and did not influence rhTRAIL sensitivity (figures 1 and 2). Therefore, the regulation of DR4 and DR5 expression by β -catenin does not seem to be mediated through TCF-4.

In summary, the results from the cell line models show that down-regulation of β -catenin leads to reduced DR4 and DR5 expression and reduced sensitivity to rhTRAIL-induced apoptosis within 48 hours. This effect does not seem to be mediated through the TCF-4 transcription factor.

β -catenin expression in colon adenomas and carcinomas is associated with DR4, but not DR5 expression

To further investigate the role of β -catenin in the regulation of DR4/5 expression in-vivo, relative expression patterns of β -catenin, DR4 and DR5 were investigated in patient-derived colorectal adenomas and carcinomas using immunohistochemistry. Cytoplasmic staining and cytoplasmic plus nuclear staining of β -catenin were more frequently observed in carcinomas (100 % and 49 % of the tumours respectively) than in adenomas (90 % and 15 % respectively) indicating increasing activation of the Wnt pathway during the adenoma carcinoma sequence as expected. Consistent with earlier results, the mean percentage of DR4 and DR5 positive cells per tumour was higher in carcinomas than in adenomas (89 % vs. 51 %, $p < 0.001$ and 94 % vs. 83 %, $p < 0.001$, respectively).

Subsequently, the results of the β -catenin, DR4 and DR5 stainings were combined. As shown in figure 3, more immunohistochemical DR4 staining was observed in colorectal adenomas with nuclear β -catenin staining than in those without (61 % vs. 46 %, $p < 0.01$). In the multivariate analysis the association between nuclear β -catenin staining and DR4 staining ($p = 0.001$) in adenomas was shown to be independent of size, degree of dysplasia and growth type of the adenoma and age of the patients. More DR5 staining was observed in colorectal adenomas with cytoplasmic β -catenin and those with cytoplasmic and nuclear staining than in those with membranous staining only (85 % vs. 70 %, $p = 0.002$ and 83 % vs. 70 %, $p = 0.015$ respectively).

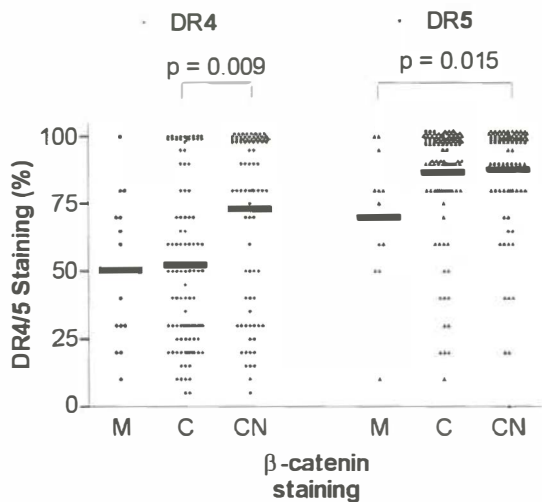


Figure 3: Percentage of DR4 and DR5 positive cells in human colorectal adenomas as determined in adenomas with membranous staining only (M), with cytoplasmic staining only (C) and cytoplasmic and nuclear staining (CN) of β -catenin. Values are expressed as individual values (dots) and the mean staining percentages (lines).

In carcinomas, cytoplasmic β -catenin staining was largely homogenous with foci of nuclear staining and all tumours were almost entirely DR4 and DR5 positive. It was therefore not possible to analyze an association between β -catenin and DR4/5 staining in carcinomas separately.

β -catenin co-localizes with DR4 and DR5

To further examine this relationship, the co-localization of β -catenin and DR4 and DR5 expression within small sub-sections of the adenomas was investigated in serial slides of the smallest adenomas. Sub-sections with increased intensity of cytoplasmic β -catenin staining compared to surrounding tissue and/or nuclear staining co-localized with sub-sections of increased DR4 and DR5 staining in 12/13 adenomas (figure 4, table 1). This indicates an association between β -catenin and both DR4 and DR5 expression.

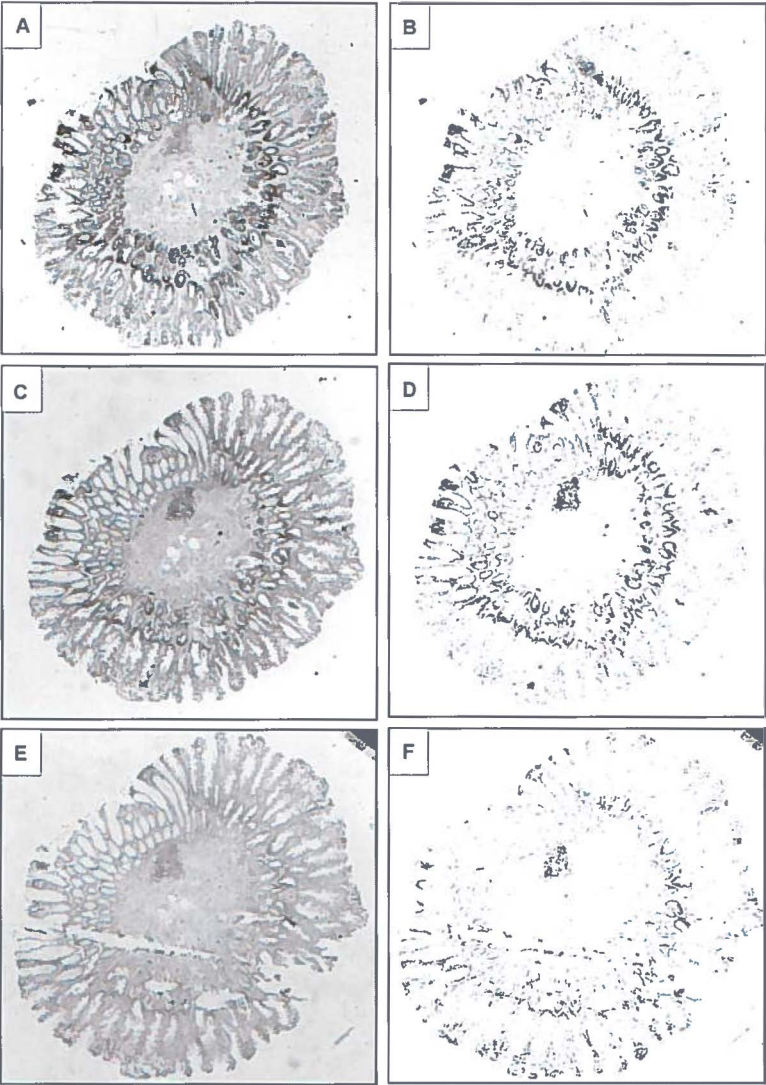


Figure 4: β -catenin (A, B), DR4 (C, D) and DR5 (E, F) stained tissue sections of an adenoma showing co-localization of staining, before (A, C, E) and after (B, D, F) accentuation of contrast. Original magnification 25x. See appendix 2, page 180 for colour figure.

The majority (78 %) of grid-sections where DR4 and DR5 co-localized showed increased β -catenin staining suggesting a role for β -catenin as a common regulator of both receptors.

The McNemar test showed that there is discordance between stainings, significant in 9/13 cases for β -catenin with DR4 and in 8/13 cases for β -catenin and DR5. This was due to the fact that positive β -catenin staining was more common than positive DR4 or DR5 staining and therefore the expression patterns of these stainings can never be identical. Consequently, maximum kappa values will always be smaller than one, for this data set. In summary, the immunohistochemistry results show that increased cytoplasmic and/or nuclear staining of β -catenin is associated with increased expression of the TRAIL receptors DR4 and DR5.

Table 1: Co-localization of β -catenin, DR4 and DR5 staining as determined in all equal grid sections containing colon epithelium in 13 adenomas.

Protein 1		Protein 2		Overlap	Kappa value ³	p value
name	squares ¹	name	squares ¹	squares ²	(range)	
β -catenin	1126	DR4	809	531	0.39 (CI 0.35-0.43)	p < 0.001
β -catenin	1126	DR5	753	474	0.37 (CI 0.33-0.40)	p < 0.001
DR4	809	DR5	753	429	0.44 (CI 0.40-0.49)	p < 0.001

¹The number of positive grid sections in each category is shown. Total grid sections = 4052. ²Overlap is the number of grid sections with positive staining for protein 1 and protein 2. ³Kappa = 0 if overlap is random and Kappa = 1 if both stainings are identical.

Discussion

This study shows that the expression levels of the pro-apoptotic TRAIL receptors, DR4 and DR5, can be regulated by β -catenin. In colon adenoma and carcinoma cell line models down-regulation of β -catenin led to lower membranous expression of DR4 and DR5 and, concomitantly, a lower TRAIL sensitivity. These results were confirmed in human colorectal adenomas where

aberrant β -catenin staining was associated with, and co-localized with, increased DR4 and DR5 staining.

The best known role of β -catenin in colon cancer is activation of Wnt signalling. Activation of the Wnt pathway, as determined through expression of TCF-4 target genes, is seen in early adenomas and increases during colorectal carcinogenesis⁷. The influence of β -catenin on DR4 and DR5 expression suggests that these receptors could be direct or indirect targets of TCF-4 signalling. There are, however, no TCF-4 consensus binding sequences (5'-CCTTTGATC-3') in the promoter regions of DR4 and DR5 and micro-array studies comparing gene expression patterns between cells with active and inactive Wnt signalling did not identify the TRAIL receptors DR4 and DR5 as TCF-4-target genes¹⁵⁻¹⁸. This is in line with our cell line results indicating that inhibition of TCF-4-signaling does not influence expression levels of DR4 and DR5 or TRAIL sensitivity. The TCF-4-target gene *c-myc* has been associated with DR5 expression. *C-myc* expression levels, however, are influenced through many other mechanisms besides aberrant Wnt signalling¹⁹⁻²². It seems that the effect of Wnt-signalling inhibition only on *c-myc* is not sufficient to influence TRAIL receptor expression. *C-myc* also regulates p21 expression, which has also been shown to influence sensitivity to rhTRAIL. Since the p21 protein was upregulated both after inhibition of β -catenin and after induction of dnTCF-4 (data not shown), with different effects of each on TRAIL receptor expression, an effect mediated through p21 also seems unlikely. These results indicate that the explanation for the association between β -catenin and DR4 and DR5 should be sought in TCF-4-independent functions of β -catenin such as cell-adhesion and activation of other transcription factors²³.

It is known that different members of the TCF-family have common functions but are not fully interchangeable²⁴. Besides TCF-4, TCF-1 and LEF-1 are also present in colorectal cancer tissue²⁴. TCF-1 and LEF-1, which are not inhibited by dn-TCF-4, also have β -catenin binding domains and could therefore be influenced by β -catenin. Furthermore, β -catenin plays an important role in cell-adhesion and is found at the adherens junctions in a complex also including E-cadherin. Interestingly, both the E-cadherin-catenin complex and the pro-apoptotic TRAIL receptors have been reported to be located in so-called lipid rafts at the cell membrane^{25,26}. It is tempting to speculate that changes in the E-cadherin-catenin complexes due to changes in β -catenin expression could influence TRAIL receptor expression. Furthermore, β -catenin can also interact with other proteins, such as the growth factor receptors c-met and c-erbB2, the

mucin MUC1 and the FOXO transcription factors, which could potentially influence regulation of DR4 and DR5²⁷⁻³⁰.

The signal recognition particle has previously been shown to play an essential role in the trafficking of DR4 to the cell surface and, furthermore, DNA damage, oncogenic mutations leading to ras activation and expression of wild-type p53 protein have previously been described as regulators of DR4 and DR5 expression³¹⁻³⁴. Our results indicate that β -catenin can be added to this list. In our cell line model, short-term suppression (48 hours) of β -catenin with siRNA resulted in approximately 30% reductions in DR4/5 levels at the cell membrane. The association between β -catenin and DR4/5 levels was even more pronounced in-vivo in human colorectal adenomas, where a clear co-localization of β -catenin positive cells and DR4/5 positive cells was observed. This may be related to the longer time-frame during which β -catenin is differentially expressed in colorectal adenomas cells in-vivo. Alternatively, overexpression of β -catenin is accompanied by changes in other factors affecting DR4/5 expression during carcinogenesis, thus short-term downregulation of β -catenin may not be sufficient to completely reverse this process. This knowledge contributes to our understanding of the physiological role of the TRAIL receptors in normal and malignant tissue, although it remains unclear whether the increased TRAIL receptor expression in colorectal tumours contributes to tumour growth or is merely a consequence of increased levels of β -catenin. An understanding of TRAIL receptor regulation in both malignant and normal tissue is necessary to be able to predict the efficacy and toxicity of rhTRAIL based therapy in different settings. The presence of the association between β -catenin and DR4/5 expression in a large panel of colorectal tumours such as those described in this paper could have interesting clinical implications. Our results indicate that caution is needed when combining rhTRAIL with drugs which target endogenous β -catenin since these drugs could effect membrane expression levels of DR4/5 and thus influence rhTRAIL sensitivity. This is important since rhTRAIL and agonistic TRAIL receptor antibodies have reached phase I/II clinical trials and are being combined with other therapeutic agents in subsequent phases of clinical testing^{12,35}.

In conclusion, we have demonstrated that the gradual increase in TRAIL-receptor expression during colorectal carcinogenesis is at least partially mediated through increased β -catenin expression. This effect seems to be independent of TCF-4-signaling.

Acknowledgements

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Chapter 5

Modulation of rhTRAIL-induced apoptosis by aspirin in colorectal cancer: a role for TCF-4

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Abstract

Background: TNF-related apoptosis inducing ligand (TRAIL) is a potential novel anti-cancer drug, which can induce apoptosis in human colorectal carcinoma cell lines. However, not all colon cancer cell lines are sensitive to recombinant human (rh)TRAIL and acquired resistance to rhTRAIL can occur. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have a chemopreventive effect in colorectal cancer and can enhance TRAIL-induced apoptosis *in vitro*. The aim of this study was to investigate whether aspirin sensitizes colon cancer cells to TRAIL-induced apoptosis and to determine whether Wntless-Int (Wnt) signalling is involved.

Methods: Human colon cancer cell lines with varying sensitivity to rhTRAIL, with acquired resistance to rhTRAIL or with doxycyclin-inducible expression of dominant negative T-cell factor-4 (dnTCF-4, to inhibit Wnt signalling) were used. The cell lines were exposed to aspirin (0-10 mM) and/or rhTRAIL (0-1.0 µg/ml). Apoptosis and caspase activation were evaluated using fluorescence microscopy, immunocytochemistry for M30 and fluorometric caspase assays. Cell membrane expression levels of the TRAIL receptors were measured using flow cytometry.

Results: Aspirin sensitized all cell lines to rhTRAIL-induced apoptosis in a dose-dependent manner. Addition of 10 mM aspirin led to a 2 – 9 fold increase in rhTRAIL-induced apoptosis measured using fluorescence microscopy. Increased apoptosis was confirmed by a greater percentage of M30 positive cells and increased caspase-3 activation. Overexpression of dnTCF-4 completely blocked the sensitizing effect of aspirin on rhTRAIL induced apoptosis.

Conclusion: Aspirin sensitizes colon cancer cell lines with intrinsic or acquired TRAIL-resistance to rhTRAIL-induced apoptosis and this effect is mediated through Wnt signalling.

Introduction

Recombinant human TNF-related apoptosis inducing ligand (rhTRAIL), currently under evaluation as an anti-cancer drug, induces apoptosis in human colon carcinoma cell lines ¹. RhTRAIL induces apoptosis by binding to its pro-apoptotic death receptors, DR4 and DR5. This induces the formation of a death-inducing signalling complex (DISC) which results in activation of procaspase-8, initiating a cascade of events resulting in apoptosis. RhTRAIL does not induce apoptosis in normal cells *in vitro* and *in vivo* and phase I/II trials with TRAIL-based drugs currently show favourable profiles ². However, both intrinsic and acquired resistance to rhTRAIL have been described in colon cancer cell lines and resistance to rhTRAIL may potentially limit its clinical use ³. Therefore, enhancement of TRAIL-induced apoptosis using modulatory drugs in the development of TRAIL-based therapies is of interest.

Non-steroidal anti-inflammatory drugs (NSAIDs) can prevent or delay colorectal cancer development and can be used to enhance TRAIL-induced apoptosis ⁴. These drugs are widely prescribed as painkillers and anti-inflammatory drugs and their effect is based on the inhibition of the COX enzymes which are involved in prostaglandin synthesis. In cancer, NSAIDs exert their effect through COX-dependent mechanisms, such as increased apoptosis and inhibition of both proliferation and angiogenesis, as well as COX-independent mechanisms ⁵. An important COX-independent mechanism is the inhibition of Wntless–Int (Wnt)-pathway signalling. In colorectal cancer somatic mutations in the *adenomatous polyposis coli* (APC) gene and the *CTNNB1* (β -catenin) gene can both lead to Wnt-pathway activation. This occurs due to accumulation of β -catenin protein eventually leading to enhanced activation of the T-cell factor-4 (TCF-4) transcription factor, resulting in the transcription of genes associated with carcinogenesis. NSAIDs are known to attenuate Wnt signalling through a number of different mechanisms including reductions in β -catenin levels and interruption of the β -catenin/TCF-4 interaction ^{6,7}.

The aim of this study was to investigate whether the NSAID acetylsalicylic acid (ASA or aspirin) sensitizes colon cancer cell lines to TRAIL-induced apoptosis and to investigate the role of Wnt pathway signalling in this sensitization.

Materials and methods

Cell culture

The colon cancer cell lines CACO-2, LOVO, SW-948, SW948-TR, Ls174T and Ls174T-dnTCF-4 cell lines were used. CACO-2 and SW948 are mismatch repair proficient cell lines while LOVO and Ls174T are mismatch repair deficient cell lines. CACO-2 and LOVO were cultured in RPMI 1640 medium (Invitrogen, Leek, the Netherlands) supplemented with respectively, 13% and 10% foetal calf serum (FCS, Invitrogen) at 37 °C in a humidified atmosphere with 5% CO₂. SW948 was cultured in Leibovitz L15-RPMI (1:1) enriched with 10% FCS, 0.05 M pyruvate, 0.1 M glutamine and 0.025% β-mercaptoethanol at 37 °C in a humidified atmosphere with 5% CO₂. The SW948-TR is a TRAIL-resistant sub-cell line of the SW948⁸. SW948 is very sensitive to rhTRAIL (inhibitory concentration resulting in 50% growth inhibition (IC₅₀): 0.007 µg/ml), LOVO is intermediately sensitive (IC₅₀: 0.04 µg/ml) and both CACO-2 and SW948-TR are resistant to rhTRAIL. The Ls174 colon cancer cell lines (Ls174T-dnTCF-4 and Ls174T) contain doxycyclin-inducible expression plasmids and were kindly provided by H. Clevers MD, PhD⁹. The “Ls174T-dnTCF-4” has a doxycyclin-inducible gene encoding N-terminally truncated versions of TCF-4. These dominant-negative TCF-4 (dnTCF-4) proteins do not bind β-catenin and act as potent inhibitors of TCF signalling. In this cell line model it is possible to down-regulate Wnt pathway activity at the TCF-4 level. The “Ls174T” is stably transfected with an empty vector containing only the doxycyclin responsive promoter as control¹⁰. The cell lines were cultured in RPMI enriched with 5% FCS at 37 °C in a humidified atmosphere with 5% CO₂. The cells were harvested by treatment with trypsin for 5-10 min at 37 °C. The cells were put under selection with blasticidin (Invitrogen) 10 µg/ml and zeocin (Invitrogen) 500 µg/ml for one week every month. To induce dnTCF-4 expression doxycyclin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the medium at a final concentration of 1 µg/ml.

Reagents

RhTRAIL was produced non-commercially in co-operation with IQ-Corporation (Groningen, the Netherlands) as described previously¹¹. The following concentrations and lengths of rhTRAIL incubation were used: LS174T (1 µg/ml, 5 hours), CACO-2 and SW948-TR (0.1 µg/ml, 24 hours), LOVO (0.01 µg/ml, 24 hours) and SW948 (0.005 µg/ml, 24 hours). A stock solution of aspirin 1 M (Sigma-Aldrich) in alcohol 100% was prepared. The aspirin stock solution was added to the cell culture medium to achieve the appropriate concentrations (0-

10 mM). The medium was then adjusted to pH 7.4 and filtered. Control cells were treated with corresponding amounts of alcohol 100% to adjust for the alcohol in the aspirin stock. The CACO-2 cell line was treated with aspirin for 48 hours and all other cell lines for 24 hours. A stock solution of indomethacin 50 mM (Sigma-Aldrich) in alcohol 100% was prepared. The indomethacin stock solution was added to the cell culture medium to achieve the appropriate concentrations (0-400 μ M). A stock solution of celecoxib 20 mM (Pfizer, Capelle a/d IJssel, The Netherlands) in dimethyl sulfoxide (DMSO) 100% was prepared. The celecoxib stock solution was added to the cell culture medium to achieve the appropriate concentrations (0-100 μ M). For indomethacin and celecoxib experiments control cells were treated with corresponding amounts of alcohol or DMSO, respectively. All incubations were performed at 37 °C and 5% CO₂ in a humidified atmosphere.

Quantification of apoptosis after rhTRAIL and/or aspirin treatment

For all cell lines 10,000 cells were seeded in 96-well plates. The cells were incubated with aspirin and/or rhTRAIL and harvested as described above. The Ls174 transfectant cell lines were induced using doxycyclin 24 hours prior to treatment. Apoptosis was identified by staining nuclear chromatin with acridine orange (10 μ g/ml) and identifying the appearance of apoptotic bodies and/or chromatin condensation by fluorescence microscopy. Apoptosis was expressed as percentage apoptotic cells in a culture. Immunocytochemistry for M30 was additionally used to quantify apoptosis. The M30 antibody (Cell Signalling Technology, Leusden, The Netherlands) binds to a caspase-mediated cleavage product of the epithelial marker cytokeratin 18¹². Cells were incubated as described above and cytopspins were made. Cytopspins were fixed in 2-propanone (Genfarma, Maarsen, The Netherlands) at room temperature and permeabilised in phosphate-buffered saline containing 0.5% Triton-X100 (Sigma-Aldrich) for 10 min. M30 antibody (1:100) was applied to the sections for 60 min at room temperature, followed by incubation with rabbit-anti-mouse peroxidase conjugated antibody (DAKO, Glostrup, Denmark) and a tertiary swine-anti-rabbit peroxidase-conjugated antibody (DAKO). The staining was visualized using 3-amino-9-ethylcarbazole and counterstained with hematoxylin. M30 positivity was identified as brown cytoplasmic staining. At least 300 cells were counted and the percentage of apoptotic cells was determined. All experiments were performed in triplicate.

Caspase enzyme activity after rhTRAIL and/or aspirin treatment

The cells were incubated with aspirin and/or rhTRAIL in 6-wells plates and harvested as described above. Activity of caspase-3 was assayed according to the manufacturer's instructions using the caspase-specific fluorescence peptide substrate Ac-DEVD-AFC (Biomol Tebu-bio, Heerhugowaard, the Netherlands). Fluorescence from free 7-amino-4-trifluoromethyl coumarin was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, the Netherlands) using a 380 nm excitation and a 508 nm emission wavelength. Relative caspase activity was obtained by comparing the treated to the untreated samples. All experiments were performed in triplicate.

Analysis of membrane expression of TRAIL-receptors using flow cytometry

The cells were incubated with aspirin and/or rhTRAIL in 6-wells plates and harvested as described above. Cells were resuspended in flow cytometry medium (phosphate-buffered saline, 2% FCS, 0.1% sodium azide). Analysis of TRAIL-receptor membrane expression was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL, USA) and cells were stained as described earlier ¹³. The following antibodies were used: huTRAILR1-M271 for DR4, huTRAILR2-M413 for DR5, huTRAILR3-M430 for DcR1 and huTRAILR4-M444 for DcR2 (all gifts from Amgen, Seattle, WA, USA). Membrane receptor expression is shown as mean fluorescence intensity of all analyzed cells. All experiments were performed in triplicate.

Statistical analysis

SPSS for Windows software (SPSS Inc., Chicago, IL, USA) was used in all statistical analyses. The Student's-t-test was used to determine differences between cell lines exposed to various conditions. P values of < 0.05 were considered significant.

Results

Aspirin sensitizes cells to rhTRAIL induced apoptosis

Aspirin sensitized all colon cancer cell lines to rhTRAIL induced apoptosis in a dose-dependent manner, inducing a 2 – 9 fold increase in rhTRAIL-induced apoptosis (figure 1A-D). These results demonstrate that it is possible to sensitize cell lines with intrinsic rhTRAIL-resistance (CACO-2) or acquired

rhTRAIL-resistance (SW948-TR) to rhTRAIL-induced apoptosis as determined with fluorescence microscopy.

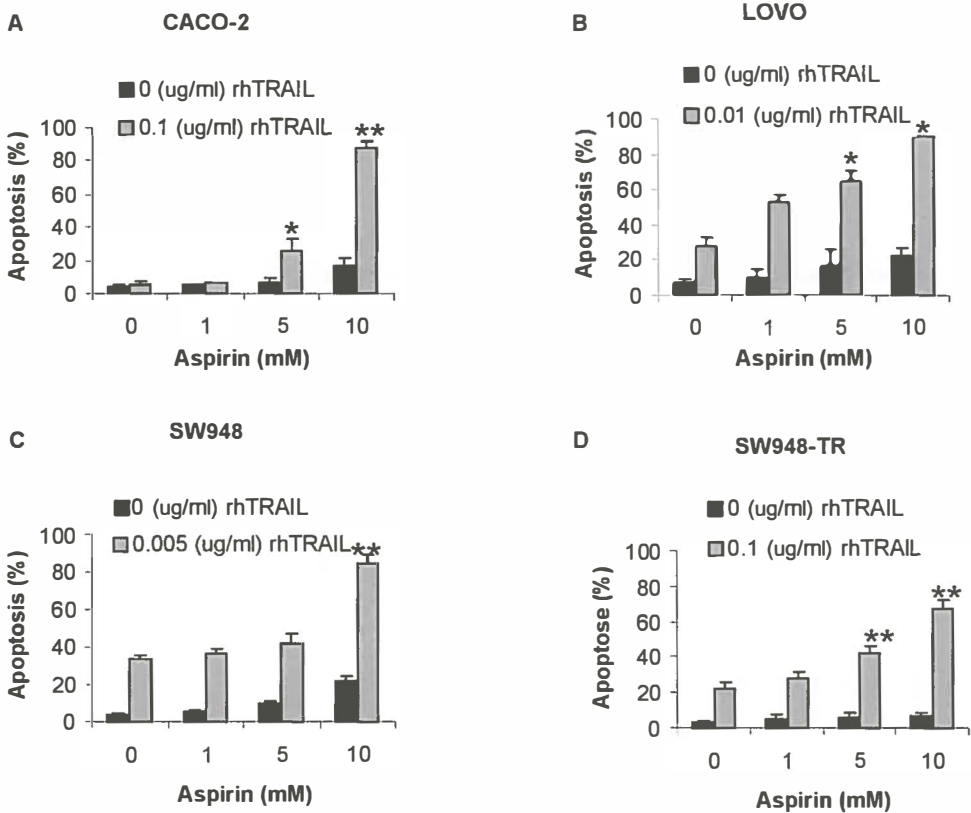


Figure 1: Apoptosis, measured using fluorescence microscopy, in the colon cancer cell lines CACO-2 (A), LOVO (B), SW948 (C) and SW948-TR (D) following incubation with aspirin (0-10 mM) in combination with rhTRAIL (0-0.1 μ g/ml). Values are the mean \pm standard deviation of three independent experiments. * $p < 0.05$ and ** $p < 0.001$ for aspirin and rhTRAIL compared to rhTRAIL only.

In the rhTRAIL-sensitive and the intermediately sensitive cell lines SW948 and LOVO incubation with aspirin also increased rhTRAIL-induced apoptosis. These apoptosis inducing effects were confirmed by observations of increased caspase-3 activation (figure 2A) and an increased percentage of M30 positive cells (figure 2B+C) after incubation with the combination of aspirin and rhTRAIL. Celecoxib (0-100 μ M) also sensitized these cell lines to TRAIL-

induced apoptosis in a similar fashion (data not shown). Indomethacin (0-400 μ M), however, did not increase rhTRAIL-induced apoptosis (data not shown).

Aspirin does not influence TRAIL-receptor expression

Changes in the expression of the TRAIL-receptors did not explain the increased rhTRAIL-induced apoptosis by aspirin. Incubation with aspirin for 24 hours led to a two-fold increase in DR5 membrane expression in the LOVO cell line. In the other cell lines, however, aspirin did not influence the membrane expression levels (figure 3).

Modulation of rhTRAIL-induced apoptosis by aspirin is mediated through TCF

In the LS174T cell lines aspirin also sensitizes cells to rhTRAIL induced apoptosis as shown in figure 4A. When dnTCF-4 expression was induced aspirin no longer had any effect on rhTRAIL-induced apoptosis (figure 4B). The observed effect in dnTCF-4 expressing cells was not due to doxycyclin treatment as the results with the doxycyclin treated control cell line demonstrate (figure 4A). These results indicate that the effects of aspirin on rhTRAIL-induced apoptosis are mediated through the TCF transcription factor.

Discussion

Intrinsic and acquired resistance to rhTRAIL-induced apoptosis have both been demonstrated in tumour cell lines and could potentially limit the efficacy of TRAIL-based drugs in the clinical setting. In this study we show that it is possible to sensitize human colon cancer cell lines to rhTRAIL-induced apoptosis using aspirin and that this effect is dose-dependent and mediated through the TCF transcription factor.

In colon cancer cell lines with intrinsic and acquired resistance to rhTRAIL-induced apoptosis, aspirin restored rhTRAIL-sensitivity and in the rhTRAIL-sensitive cell lines aspirin reduced the concentrations of rhTRAIL needed to induce apoptosis. A previous study demonstrated that aspirin reduces cell survival of human colon carcinoma cells in a dose-dependent manner in combination with rhTRAIL¹⁴.

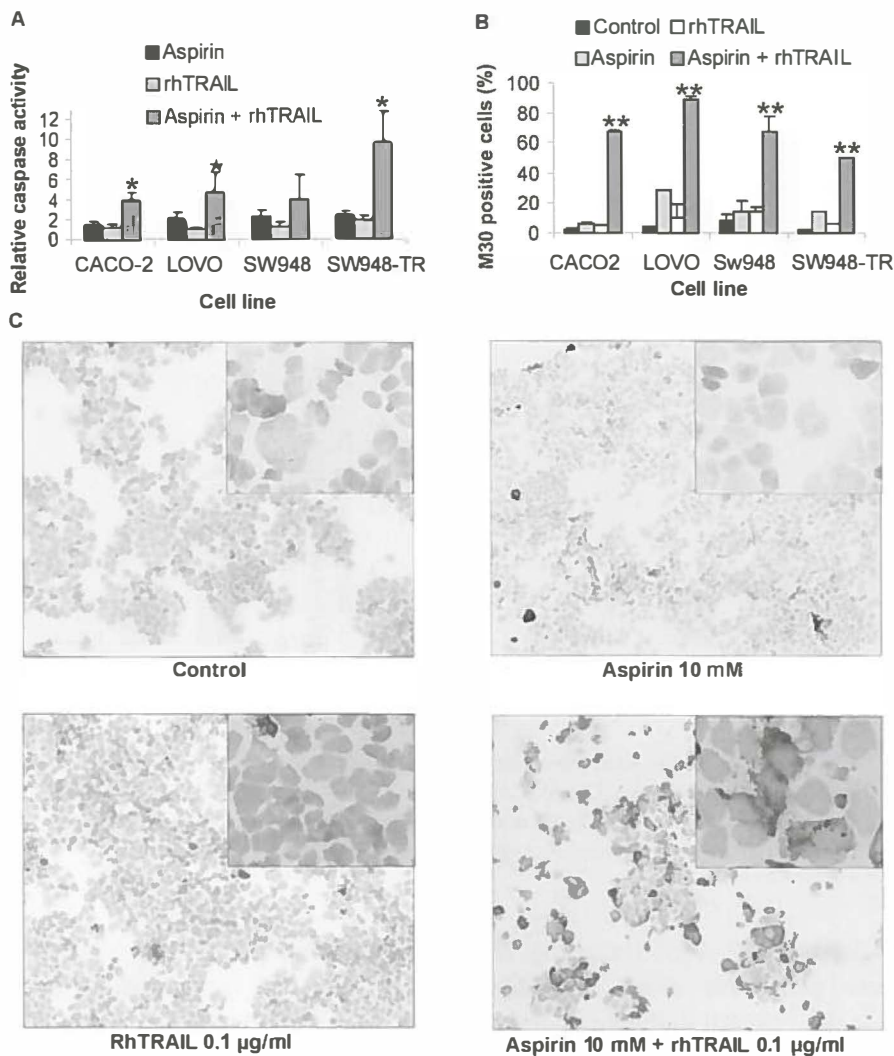


Figure 2: Caspase-3 activity in carcinoma cell lines after incubation with combinations of aspirin (10 mM) and rhTRAIL (0.005 – 0.1 µg/ml) determined using a fluorimetric caspase-3 assay (A). Relative caspase activity is the ratio of treated to untreated cells. Values are the mean \pm standard deviation of three independent experiments. * $p < 0.05$ for aspirin and rhTRAIL compared to rhTRAIL only. Degree of apoptosis (%) in CACO-2, LOVO, SW948 and SW948-TR, after treatment with rhTRAIL and/or aspirin, as determined by immunocytochemistry for M30 (B). Values are the mean \pm standard deviation of three independent experiments. * $p < 0.05$ for aspirin and rhTRAIL compared to rhTRAIL only. Apoptosis in the CACO-2 cell line after incubation with aspirin and/or rhTRAIL measured using M30 staining (C). M30 positive cells stain brown. Original magnification main figures: 40x. Original magnification inserts: 400x. One representative of at least three independent experiments is shown. See appendix 2, page 181 for colour figure.

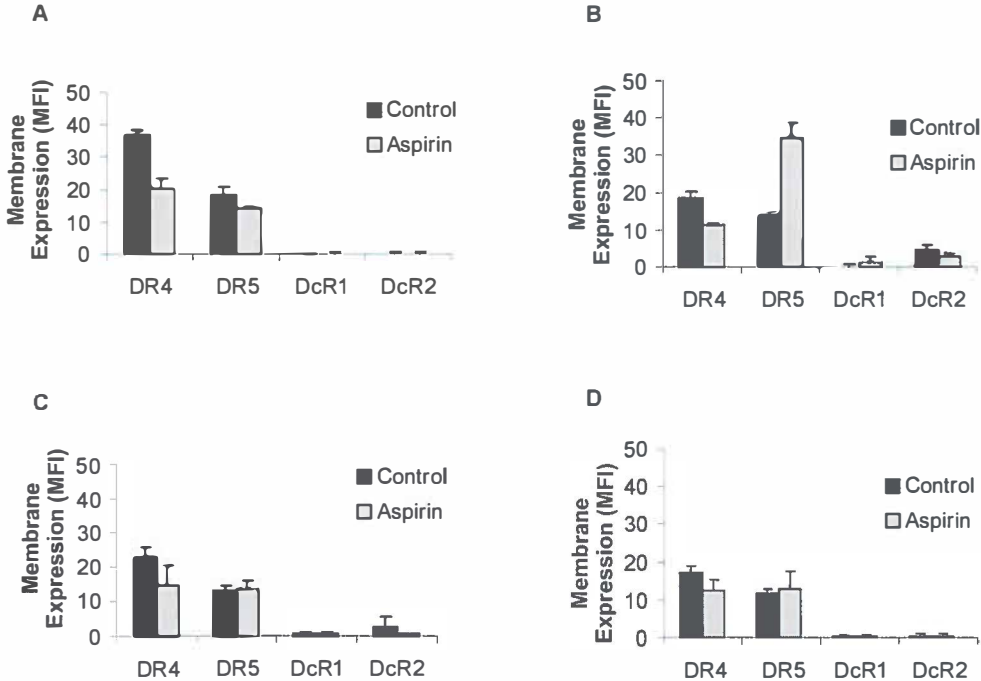


Figure 3: TRAIL-receptor membrane expression after 24 hours incubation with aspirin 5 mM (SW948) or 10 mM (CACO-2, LOVO, SW948-TR). CACO-2 (A), LOVO (B), SW948 (C), SW-948-TR (D). Values are expressed as the mean fluorescence intensity (MFI) \pm standard deviation of three independent experiments.

This is, however, not surprising since aspirin reduces proliferation of colon cancer cells. We show that, besides reducing cell proliferation, aspirin enhances rhTRAIL-induced apoptosis. The enhancement of apoptosis in all tumour cell lines studied indicates that the effects of aspirin on rhTRAIL-induced apoptosis are not tumour cell line specific. Effects were seen in mismatch repair proficient (CACO-2, SW948) and mismatch repair deficient (LOVO, Ls174T) cell lines. Various other non-selective NSAIDs and selective COX-2 inhibitors have been shown to potentiate rhTRAIL-induced apoptosis in human colon carcinoma cells, as well as in cervical cancer and non-small cell lung cancer cell lines¹⁵⁻¹⁷. We also observed that the specific COX-2 inhibitor celecoxib, but not the non-selective NSAID indomethacin, sensitized colon cancer cells to rhTRAIL-induced apoptosis. This indicates that enhancement of rhTRAIL-induced apoptosis is not a class effect of NSAIDs but does occur in both selective and non selective NSAIDs.

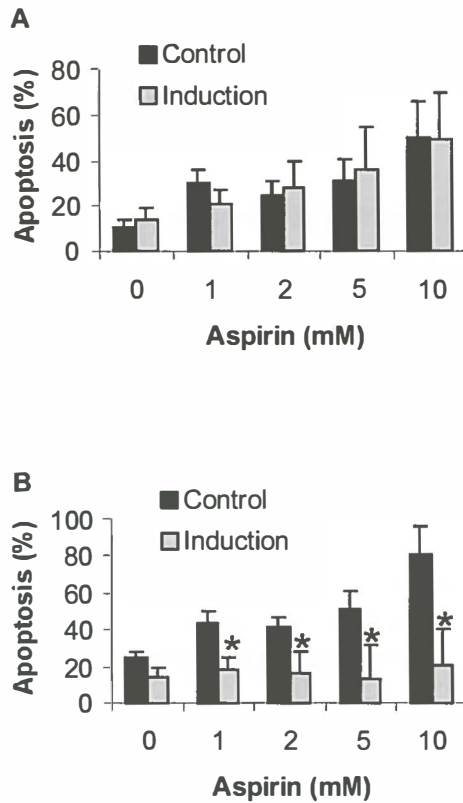


Figure 4: Apoptosis in the LS174 cell lines (with and without doxycyclin induction) after incubation with aspirin (10 mM) and rhTRAIL (1 μ g/ml). Apoptosis measured using fluorescence microscopy. (A) Control cell line LS174T. (B) Cell line LS174-dnTCF-4 with dominant negative TCF-4 expression after doxycyclin treatment. Values are the mean \pm standard deviation of three independent experiments. * $p < 0.05$ in LS174-dnTCF-4 treated with aspirin and rhTRAIL: not induced vs. induced with doxycyclin.

In our panel of colon cancer cell lines modulation of rhTRAIL-induced apoptosis by aspirin could not be explained by increased membranous expression of the pro-apoptotic TRAIL-receptors DR4 and DR5. Sulindac sulfide and the selective COX-2 inhibitors celecoxib and SC-236 all increase DR5 expression levels⁴. However, these increases have only been demonstrated at mRNA or total cellular protein levels and increases in membrane DR5 expression by

NSAIDs have, in accordance with our results, never been found. Enhancement of rhTRAIL-induced apoptosis does involve clustering of the TRAIL-receptor DR5 at the cell surface and redistribution into cholesterol-rich and ceramide-rich domains known as caveolae or lipid rafts ¹⁷. Further studies are needed to determine whether NSAIDs differentially influence TRAIL-receptor localization and thus sensitization to rhTRAIL-induced apoptosis without affecting the overall TRAIL receptor membrane expression levels.

Aspirin no longer enhanced rhTRAIL-induced apoptosis when dnTCF-4 was expressed. One possible explanation is that the effect of aspirin is mediated solely through downregulation of TCF-4 signalling. However, the expression of dnTCF-4 itself did not enhance rhTRAIL-induced apoptosis in our model, making this explanation unlikely. Therefore, although aspirin itself downregulates TCF-4 signalling in colon cancer cells, some remaining TCF-4 signalling is necessary to obtain aspirin-mediated enhancement of rhTRAIL-induced apoptosis ⁷. This is in agreement with the fact that the enhancement of rhTRAIL-induced apoptosis by aspirin has only been demonstrated in colon and prostate cancer cell lines, both known for their active Wnt-signalling ¹⁴. Additional studies are required to investigate which components of the TRAIL apoptosis pathway are modulated by both aspirin and Wnt signalling, either directly or indirectly, to determine why these effects occur.

TRAIL resistance is encountered in in vitro studies and therefore the possibility to increase sensitivity to rhTRAIL and to circumvent TRAIL-resistance using aspirin is very interesting. Increased TRAIL-receptor expression and localization to lipid-rafts, as well as the downregulation of inhibitors of TRAIL-induced apoptosis such as Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP) have been associated with sensitisation to rhTRAIL by NSAIDs ⁴. Furthermore, in the modulation of rhTRAIL-induced apoptosis by other agents, such as interferon- γ , caspase-8 has been shown to play an important role ¹⁸. The exact mechanisms behind enhanced rhTRAIL-induced apoptosis by aspirin need to be unravelled to determine optimal modulation strategies in patients.

In conclusion, we have demonstrated that it is possible to enhance rhTRAIL-induced apoptosis using aspirin in a panel of colon cancer cell lines with varying sensitivity to rhTRAIL and that Wnt signalling plays a role in increasing rhTRAIL-sensitivity. This synergistic effect could potentially be exploited when TRAIL-based drugs are used in the clinical setting.

Acknowledgements

We would like to thank W Boersma-van Ek, J de Vries and E Visser-Punter for excellent technical assistance. This study was supported by grant RUG 2005-3361 (Dutch Cancer Society) and grant MLDS WS 01-31 (Dutch Digestive Diseases Foundation).

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Chapter 6

High grade dysplasia in sporadic fundic gland polyps: a case report and review of the literature

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Abstract

We present a case of fundic gland polyps (FGPs) containing high-grade dysplasia in a 68-year-old man. High-grade dysplasia, and even gastric adenocarcinoma, associated with FGPs has been described in patients with familial adenomatous polyposis (FAP) and attenuated FAP (AFAP), but never in non-FAP patients. Two colonoscopies in the past six years virtually rule out FAP and AFAP in our patient. Dysplasia in FGPs from non-FAP patients is extremely rare and until now only cases of low-grade dysplasia have been described. The literature on dysplasia in FGPs is briefly reviewed. Additional immunohistochemical investigations in this case showed nuclear staining of β -catenin, increased proliferation and apoptosis in the dysplastic areas of the FGPs. Our case suggests that the malignant potential of FGPs is not limited to FAP-associated FGPs.

Introduction

Fundic gland polyps (FGPs) are the most common type of gastric polyps. At endoscopy FGPs are seen in up to 5% of patients ¹⁻⁴. They are small, sessile polyps arising in the gastric acid secreting mucosa. Histologically, FGPs are characterized by distorted glandular architecture, usually consisting of microcysts lined with fundic type epithelial cells (chief and parietal cells) or foveolar type mucous cells ⁵. FGPs have always been considered benign lesions with no malignant potential. However, in recent years, there have been several reports of high-grade dysplasia, and even adenocarcinoma, in FGPs associated with familial adenomatous polyposis (FAP) ⁶ or attenuated FAP (AFAP) ⁷. FGPs are found in 80-93% of these patients ⁸⁻¹¹ and dysplasia has been observed in up to 54% ^{5,9,12-16}. In contrast, dysplasia in FGPs from non-FAP patients is extremely rare and only cases of low-grade dysplasia have been described. We present the case of a man with FGPs containing high-grade dysplasia, in whom FAP and AFAP were excluded.

Case report

A 68-year-old caucasian male was referred to his local hospital for complaints of food intolerance, upper abdominal pain and regurgitation. At oesophago-gastroduodenoscopy multiple polyps in the fundus and the body of the stomach were seen. There were no other abnormalities. Biopsies were taken and histopathological examination revealed that the polyps were FGPs. High-grade dysplasia was seen in two of five biopsies. *H. Pylori* was absent. The patient was referred to our hospital for further investigations. On reviewing the histopathological slides high-grade dysplasia in the FGPs was confirmed. Since high-grade dysplasia in FGPs has only been reported in patients with FAP or AFAP, the possible presence of these disorders was considered. The family history for colorectal cancer and cancer at other sites was negative. At prior colonoscopy six years earlier no colorectal polyps had been seen. Colonoscopy was repeated and was again normal. Histopathological examination of random biopsies taken during the colonoscopy did not reveal the presence of microadenomas. Follow-up oesophagogastroduodenoscopies six, twelve and eighteen months after the index endoscopy again showed multiple polyps resembling FGPs. This time, however, histopathological examination revealed FGPs without dysplasia.

For further histopathological examination the two FGPs containing dysplasia were compared with two FGPs without dysplasia and two sections of normal gastric mucosa from this patient. Hematoxylin and eosin (H&E) stained sections of FGPs showed the classical histological features, including cystically dilated fundic-type glands lined by chief and parietal cells. The FGPs containing dysplasia showed epithelial crowding with large irregular nuclei and prominent nucleoli consistent with high-grade dysplasia (figure 1) ¹⁷.

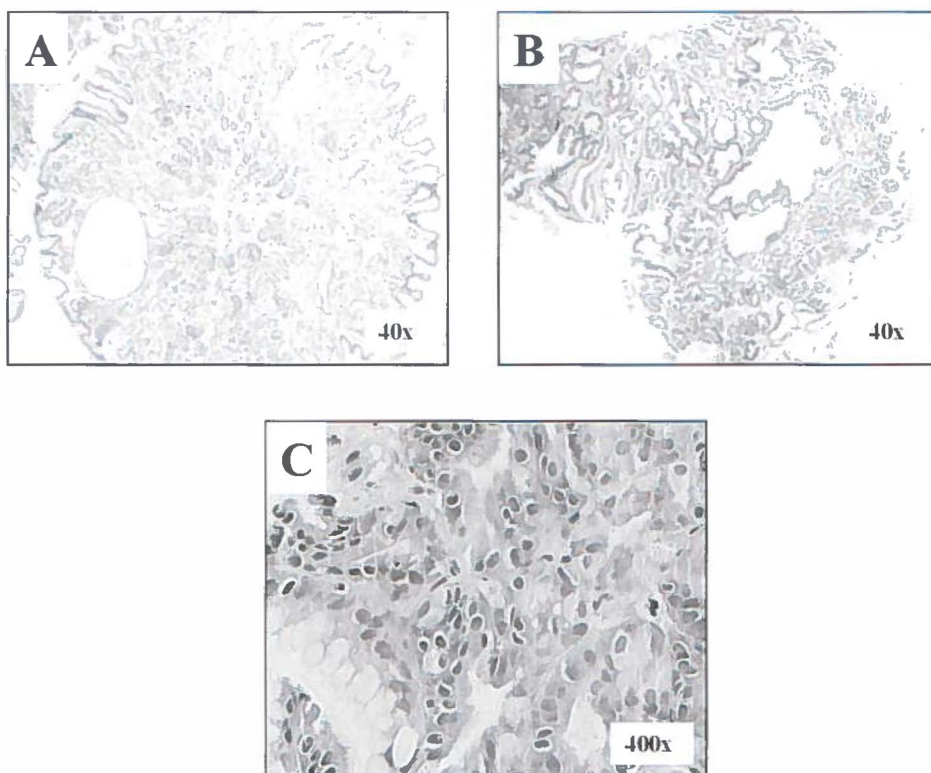


Figure 1: (A) Histopathological appearance of one of the fundic gland polyps without dysplasia (H&E, magnification x40). (B) Histopathological appearance of one of the fundic gland polyps containing high-grade dysplasia characterized by nuclear hyperchromatism and stratification and irregular size (H&E, magnification x40). (C) Detail of dysplastic area of FGP shown in B (H&E, magnification x400). See appendix 3, page 184 for colour figure.

Additional immunohistochemical staining was performed. The presence of dysplasia was associated with a higher degree of proliferation (Mib-1; 1:400, Immunotech, Marseilles, France, figure 2) and a higher degree of apoptosis (MAb M30, 1:50, Boehringer Mannheim, GmbH, Mannheim, Germany), as compared to non-dysplastic areas and normal mucosa.

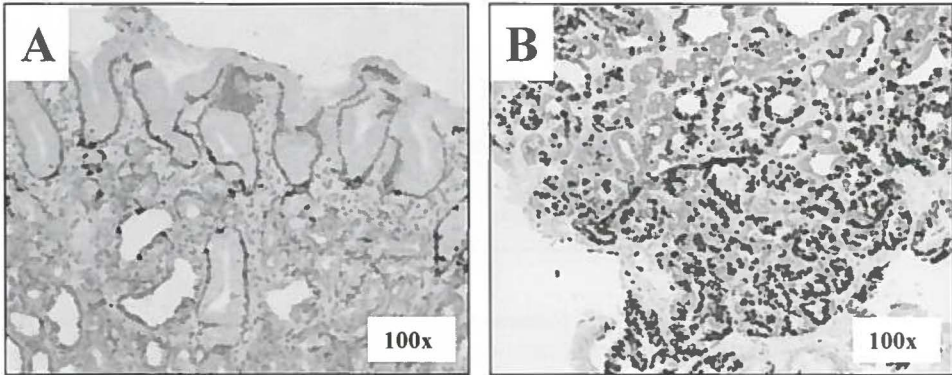


Figure 2: Immunostaining for proliferation with Ki-67. (A) One of the FGPs without dysplasia, proliferation is confined to the neck stem cell region (magnification x100). (B) One of the FGPs with high-grade dysplasia, the proliferative area extends beyond the neck stem cell region (magnification x100). See appendix 3, page 185 for colour figure.

In sporadic FGPs, a high frequency of somatic mutations in the *β-catenin* gene (*CTNNB1*) has been found while this was not observed in syndromic FGPs. Nuclear *β-catenin* staining (Transduction Laboratories, 1:1000, Lexington, KY, USA, figure 3) was seen in the dysplastic areas of the FGPs, while the non-dysplastic FGPs and normal gastric mucosa showed membranous and some cytoplasmic staining. There was expression of two target genes of the Wnt-APC-*β-catenin* pathway: c-myc (1:150, Neomarkers, Freemont, CA, USA) and cyclin D1 (1:500, clone AM-29, Zymed, San Francisco, CA, USA) in the FGPs. However, expression of these genes was not different between areas with and without dysplasia. There was no overexpression of p53 (1:400, Biogenex, San Ramon, CA, USA) in the FGPs indicating that wild-type p53 was involved in the regulation of apoptosis in these FGPs¹⁸. The expression of the mismatch repair gene MLH1 (1:500, Pharmingen, San Diego, CA, USA) was comparable

with that in the normal positive control, making loss of function of this gene unlikely.

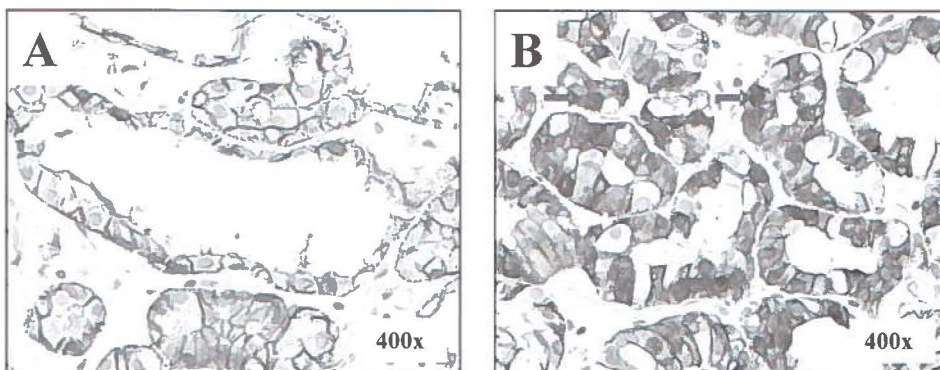


Figure 3: Immunostaining for β -catenin in the FGPs. (A) Fundic gland polyp without dysplasia showing mainly membranous and some cytoplasmic staining (magnification x400). (B) Fundic gland polyp containing high-grade dysplasia and nuclear β -catenin staining, with arrows showing examples of positively stained nuclei (magnification x400). See appendix 3, page 185 for colour figure.

Discussion

In this paper, a case of a patient with FGPs containing high-grade dysplasia is presented. FAP and AFAP were excluded in this patient. To our knowledge, high-grade dysplasia has never been reported in FGPs from non-FAP patients. Our case suggests that the possible malignant transformation of FGPs is not confined to FAP- or AFAP-associated FGPs.

Traditionally, FGPs have been regarded as benign, non-neoplastic lesions without potential for malignant transformation. However, reports of FGPs containing dysplastic foci or even adenocarcinoma, especially when associated with FAP or AFAP, have raised new interest in the pathology of FGPs^{19,20}. Goodman *et al.* described two patients with FAP who developed gastric adenocarcinoma in a background of FGPs²¹. In one patient, dysplasia was found in FGPs discontinuous with the main tumour and gastric adenomas were absent suggesting that the cancer had developed from a dysplastic FGP. Other

cases of gastric adenocarcinoma associated with FGPs have been reported in three FAP patients^{6,22,23} and two AFAP patients^{7,24}. Coffey *et al.* described a young patient with Gardner's syndrome who developed gastric adenocarcinoma in association with fundic gland polyps⁶. Zwick *et al.* and Hofgartner *et al.* described two related patients with AFAP and gastric adenocarcinoma associated with diffuse fundic gland polyposis^{7,24}. In addition, cases of FGPs with high-grade dysplasia associated with FAP and AFAP have been described^{17,23,25}. Attard *et al.* described high-grade dysplasia in FGPs in a patient with AFAP requiring prophylactic gastrectomy¹⁷. Odze *et al.* reported a patient with FAP who was found to have severe dysplasia in a large (11 x 8 x 3.5 cm) exophytic gastric tumour that appeared to arise on a background of diffuse fundic gland polyposis²³. McGarrity *et al.* described an AFAP patient with a giant FGP with extensive areas of dysplasia on a background of diffuse fundic gland polyposis²⁵.

Furthermore, there are several studies that evaluated the presence of dysplasia in FAP-associated and sporadic FGPs (summarised in table 1). Dysplasia has been found in 0-54% of FAP- or AFAP-associated FGPs and 0-5 % of sporadic FGPs. The wide range reported in syndromic FGPs might be attributed to different and inconsistent histopathological criteria that were used to establish the presence of dysplasia. Only recently, efforts have been made to develop common worldwide terminology for gastrointestinal epithelial neoplasia²⁶. Nevertheless, most studies indicate that dysplasia is encountered far more frequently in FAP-associated FGPs than in sporadic FGPs. Furthermore, the sporadic FGPs with dysplasia that have been described all concerned cases of low-grade dysplasia⁹.

Sporadic FGPs are thought to arise through mutations in the β -catenin gene (*CTNNB1*), causing activation of the Wnt-APC- β -catenin pathway^{14,27}. Activation of this pathway leads to the transcriptional activation of several target genes, including c-myc and cyclin D1, involved in the regulation of proliferative activity and apoptosis²⁸. It has recently been shown that sporadic FGPs containing low-grade or indefinite dysplasia are more likely to harbour a mutation in the *APC* gene than in the β -catenin gene²⁹.

This indicates that the sporadic FGPs containing dysplasia resemble the FAP-associated FGPs more than the non-dysplastic sporadic FGPs. We have recently demonstrated that dysplasia in both syndromic and sporadic FGPs is

associated with nuclear expression of β -catenin and increased proliferative activity and apoptosis³⁰.

Table 1. Overview of studies on the prevalence and degree of dysplasia in FAP/AFAP associated FGPs and non-FAP (sporadic) FGPs.

	FAP/AFAP				Non-FAP			
	Patients (n)	FGPs (n)	Dysplasia %	Degree	Patients (n)	FGPs (n)	Dysplasia %	Degree
Wu ⁹	24	49	25	LGD	216	270	1	LGD
Domizio ¹⁶	102	44	2	LGD	-	-	-	-
Sarre ⁴⁰	102	26	0		-	-	-	-
Bertoni ¹²	18	m.d.	44	LGD	-	-	-	-
Attard ³⁹	m.d.	13	54	1 HGD, LGD	m.d.	3	0	-
Abraham ¹³	17	41	39	1 HGD, LGD	13	13	0	-
Abraham ¹⁴	13	19	53	m.d.	40	57	0	-
Jalving ³⁰	6	9	44	1 HGD, LGD	18	19	5	LGD
Iida ¹⁰	12	12	0	-	23	23	0	-
Odze ⁵	15	15	0	-	62	62	0	-
Marcial ⁴¹	-	-	-	-	56	56	0	-
Declich ³	-	-	-	-	24	24	0	-
Torbenson ⁴²	-	-	-	-	8	62	0	-
Choudry ³²	-	-	-	-	m.d.	9	0	-

LGD: low-grade dysplasia; HGD: high-grade dysplasia; m.d.: missing data

Although this suggests a potential for malignant transformation, follow-up endoscopies in this patient have not shown signs of progression of the FGPs or the development of gastric adenocarcinoma. Sampling error may, however, obscure this development. Wei *et al.* have suggested that altered localisation of the tumour suppressor tuberlin may block nuclear translocation of β -catenin in sporadic FGPs³¹. Our observation of nuclear β -catenin staining suggests that this does not always occur, but the possible relationship between the two proteins merits further investigation.

It must be noted that our patient had received therapy with proton pump inhibitors (PPI), for approximately 10 months, prior to the index endoscopy. There have been several reports of FGPs occurring in association with PPI

therapy³²⁻³⁵. However, a large case-control study did not support this association⁴. Some studies found specific histologic differences between FGPs associated with PPI use and those not associated with PPI use^{36,37}, but others did not^{32,38}. Attard *et al.* found dysplasia in FGPs to be more common in FAP patients on long term PPI therapy than in those without therapy³⁹. In our case, the PPI therapy was discontinued at the time of the index endoscopy.

In summary, we have described a 68-year-old patient with FGPs containing high-grade dysplasia. Negative colonoscopies at 62 and 68 years of age have effectively ruled out FAP and AFAP. Our case illustrates that severe dysplasia in FGPs is not limited to syndromic FGPs. However, the question whether dysplasia really heralds malignant transformation of FGPs remains unanswered.

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Chapter 7

Dysplasia in fundic gland polyps is associated with nuclear β -catenin expression and relatively high cell turnover rates

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Abstract

Background: Fundic gland polyps (FGPs) occur in a syndromic and a sporadic form. Syndromic FGPs arise through mutations in the *adenomatous polyposis coli* (*APC*) gene whereas sporadic FGPs are caused by *β-catenin* gene mutations. Dysplasia in sporadic FGPs, found less often than in syndromic FGPs, was recently associated with *APC*- rather than *β-catenin* mutations. These data suggest different functional consequences of *APC* and *β-catenin* mutations. To investigate this hypothesis, we examined proliferative activity, degree of apoptosis, *β-catenin* expression and p53 expression in syndromic and sporadic FGPs.

Methods: Syndromic FGPs (n=9) from familial adenomatous polyposis (FAP) patients and sporadic FGPs (n=18) were studied. Proliferative activity, apoptotic cell death and expression of *β-catenin* and p53 were examined by immunohistochemistry. In FGPs containing dysplasia, areas with and without dysplasia were compared.

Results: Syndromic and sporadic FGPs without dysplasia exhibited similar proliferative activity, degree of apoptosis, *β-catenin* and p53 expression. Dysplasia was observed more often in syndromic (4/9) than in sporadic FGPs (1/18). Within FGPs containing dysplasia, dysplastic areas showed abnormal nuclear *β-catenin* staining in 3/5 cases and higher rates of cell proliferation and apoptosis than non-dysplastic areas. Overexpression of p53 was not observed.

Conclusion: The finding of similar rates of proliferation and apoptosis in syndromic and sporadic FGPs does not support the hypothesis that *APC* and *β-catenin* gene mutations have different functional consequences in FGPs. The association of dysplasia with relatively high cell turnover rates and nuclear expression of *β-catenin* indicates activation of the Wnt-APC-*β-catenin* pathway in dysplasia. The finding of dysplasia in some but not all syndromic FGPs suggests the involvement of other genes in addition to the *APC* gene in the development of dysplasia in FGPs.

Introduction

Fundic gland polyps (FGPs) are the most common gastric polyps^{1,2}. FGPs are characterized histologically by distorted glandular architecture consisting of microcysts, often lined with fundic type epithelial cells (chief and parietal cells) but occasionally with foveolar type mucous cells (figure 1)³. FGPs occur in a sporadic and a syndromic form. The syndromic form is associated with familial adenomatous polyposis (FAP) and attenuated FAP (AFAP)⁴. Fundic gland polyps are found in up to 1.9% of the general population⁵⁻⁷, in up to 84% of FAP patients⁶⁻¹³ and in 93% of AFAP patients⁴. Histologically, sporadic and syndromic FGPs are identical^{3,14}. FGPs have always been regarded as benign lesions but there have been case-reports of FGPs harbouring severe dysplasia or even gastric adenocarcinoma, particularly when associated with FAP¹⁵⁻¹⁷ or AFAP¹⁸⁻²¹. Low grade dysplasia has been observed in up to 53% of syndromic FGPs and in up to 2.3% of sporadic FGPs^{3,9,13,14,22-24}. FAP is caused by a germline mutation in the *APC* gene causing hundreds to thousands of adenomatous polyps in the colon. AFAP typically presents 10-15 years later than FAP and is associated with fewer adenomas. It is caused by germline mutations in the 5' or 3' end of the *APC* gene⁴.

Activation of the Wnt-APC- β -catenin pathway is thought to be involved in the pathogenesis of both syndromic and sporadic FGPs²⁴. In FAP, a germline *APC* mutation and subsequent somatic mutation of the second allele causes activation of this pathway²⁵. There is a high frequency of somatic mutations of the second *APC* allele in syndromic FGPs but not in sporadic FGPs^{23,26}. In sporadic FGPs, a high frequency of somatic mutations in the *β -catenin* gene (*CTNNB1*) was found while this was not observed in syndromic FGPs²⁴. Interestingly, it was recently shown that dysplasia in sporadic FGPs was more likely to be associated with *APC* alterations than with *β -catenin* mutations²⁷.

Both *APC* gene mutations and *β -catenin* gene mutations result in inadequate degradation of β -catenin causing accumulation of β -catenin in the cell cytoplasm²⁵. Following nuclear translocation of β -catenin, it binds to T-cell factors (TCFs) to become a transcriptional activator of several target genes. These target genes include *c-myc*, *cyclin-D1*, *matrilysin*, *peroxisome proliferator-activated receptor delta* and *MDR1* (multidrug resistance)²⁸. Thus, activation of the Wnt-APC- β -catenin pathway affects processes such as cell proliferation, migration, differentiation and apoptosis all of which may contribute to tumour development²⁵.

Although much has become clear about the genetic background of syndromic and sporadic FGPs, including knowledge of genetic alterations in sporadic FGPs containing dysplasia, little is known about the functional consequences of these mutations in FGPs. It has been suggested that *β-catenin* mutations are not equivalent to *APC* mutations in their functional consequences²⁴. We therefore undertook this study in a series of syndromic and sporadic FGPs, including a subset of FGPs with dysplasia, to investigate possible differences in proliferative activity, degree of apoptosis, expression of *β-catenin* and p53 expression.

Materials and methods

Case selection

Syndromic FGPs were selected from a database containing information about all patients with FAP treated at the University Hospital Groningen from 1970 to 2000. In each patient, the diagnosis FAP had been established by the presence of hundreds of adenomatous polyps found at colonoscopy at an early age, histopathological examination and *APC* gene mutation analysis. In this period, 38 patients were diagnosed with FAP. Nineteen of these 38 patients had undergone gastroduodenoscopy and FGPs had been found in nine of these 19 patients. Nine FGPs from six patients were available for immunohistochemistry. One of these patients reported previous use of proton pump inhibitors (PPI).

For comparison with the syndromic FGPs, 18 sporadic FGPs from sex- and age matched patients were selected from consecutive patients undergoing diagnostic gastroduodenal endoscopy at the University Hospital Groningen in 1999. To exclude FAP in these patients, a careful family history for colorectal cancer was taken (negative in all cases) and all clinical and endoscopic data were revised thoroughly. Sigmoidoscopy, performed in three patients, and colonoscopy, performed in two patients, revealed no abnormalities. Eight of these patients reported previous use of PPIs. All endoscopies were performed by experienced gastroenterologists.

Tissue collection and histology

Paraffin-embedded tissue specimens were retrieved from the files of the Department of Pathology, University Hospital Groningen. Histological classification of FGPs was carried out in hematoxylin and eosin (H&E) stained slides by a gastrointestinal pathologist (AK). The diagnosis FGP was made when histologic examination revealed fundic mucosa with cystically dilated glands^{14,24}. Criteria for the diagnosis of dysplasia included nuclear enlargement or pleomorphism, stratification, and hyperchromatism¹³. Dysplasia was graded as negative, low grade or high grade, according to Wu *et al*¹³.

Immunohistochemistry

For immunohistochemical staining procedures, 3 μ m-thick-sections were cut from paraffin blocks. After deparaffinisation, endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min. Proliferative activity was determined by Ki-67 immunostaining. After overnight antigen retrieval in Tris buffer (pH 9.0) at 80 °C, slides were immersed in a 1:400 solution of a mouse monoclonal antibody to Ki-67 (MIB-1, Immunotech, Marseilles, France) for 60 min. For the detection of apoptotic cells, immunohistochemistry was performed with the murine monoclonal antibody M30 (Boehringer Mannheim, GmbH, Mannheim, Germany), which reacts with a cleavage product of cytokeratin 18 (CK 18), released by activated caspase²⁹. CK 18 is a major component of the intermediate filaments of epithelial cells. For antigen retrieval, slides were immersed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven for 8 min at 700W. The primary antibody was applied to the sections for 60 min at room temperature in a 1:50 solution. For β -catenin immunostaining, antigen retrieval was performed in 0.01M citrate buffer (pH 6.0) in a microwave oven for 8 min at 700 W. A monoclonal β -catenin mouse antibody (1:1000, Transduction Laboratories, Lexington, KY, USA) was applied for 60 min. For the detection of p53, a murine monoclonal antibody that recognises both wild type and mutant p53 was applied for 60 min (1:400, clone BP-5312-1, Biogenex, San Ramon, CA, USA). Incubation with the primary antibody was followed by incubation for 30 min with a secondary rabbit-anti-mouse antibody conjugated with peroxidase (DAKO, Glostrup, Denmark). This was followed by incubation for 30 min with a tertiary goat-anti-rabbit peroxidase-conjugated antibody (DAKO) to intensify the staining reaction. Peroxidase activity was visualised with diaminobenzidine. Counterstaining was performed with hematoxylin. As negative controls, slides were immunostained in the absence of the primary antibodies. As a positive control for MIB-1, M30 and p53 staining, a section of

colon carcinoma was included. As a positive control for β -catenin staining, a section of colon carcinoma was included containing areas with membranous, cytoplasmic and nuclear staining.

Evaluation of staining

Evaluation of staining was performed by light microscopy. Sections were scanned at low power to select representative areas. Slides were evaluated by at least two independent investigators. If the evaluations did not agree the specimens were re-evaluated under a multi-headed microscope. For evaluation of MIB-1 immunostaining, at least 500 epithelial cells lining the dilated cysts were counted, according to Odze³, and at least 500 cells at the junction of the foveolar epithelium with the glandular zone, the so-called neck stem cell region. The number of positively stained nuclei was expressed as a percentage of the total number of cells counted (proliferative index). In FGPs containing dysplasia, proliferative indices were recorded separately in neck stem cell regions in dysplastic and non-dysplastic areas. The distribution of MIB-1 positive cells in FGPs was recorded according to Odze³. M30 positive cells were determined within microscope fields at 400x magnification (high power field, HPF). Results are expressed as total number of positive cells per 10 HPFs. In FGPs containing dysplasia, M30 positive cells were recorded separately in dysplastic and non-dysplastic areas. β -catenin staining was recorded as membranous, cytoplasmic or nuclear, according to Miyazawa³⁰. In FGPs containing dysplasia, patterns of β -catenin staining were compared in dysplastic and non-dysplastic areas. P53 overexpression was defined as more than 10% of cells showing strong nuclear staining in accordance with Murakami³¹. Immunohistochemical overexpression of p53 is associated with loss of tumour suppressor function³².

Statistical analysis

Patient characteristics were compared using Chi-square tests. The Mann-Whitney U test was used to assess differences in proliferative activity and degree of apoptosis between sporadic and syndromic FGPs, and between areas with and without dysplasia. Differences in β -catenin expression between FGPs with and without dysplasia were assessed using Chi-square tests. P-values under 0.05 were considered significant. SPSS for Windows software (SPSS Inc., Chicago, IL, USA) was used in all statistical analyses.

Results

Histological features

All FGPs studied showed, histologically, the typical distorted glandular architecture. This consisted of microcysts lined with chief cells, parietal cells and also foveolar type mucous cells (figure 1).

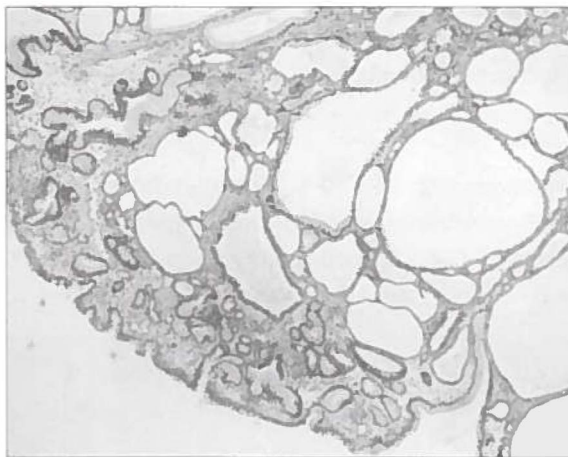


Figure 1. Typical histopathological appearance of a fundic gland polyp, showing multiple dilated cysts. See appendix 4, page 188 for colour figure.

Dysplasia, when present, involved the foveolar and surface epithelia but not the epithelium of the dilated cysts. Dysplasia was found in five FGPs, four syndromic and one sporadic FGP. The degree of dysplasia was low in four cases and high in one case, a syndromic FGP. The sporadic FGP containing dysplasia was obtained from a 52-year-old patient in whom FAP was excluded by colonoscopy. The remaining 22 FGPs were negative for dysplasia. In total, 4/9 (44%) of the FGPs from FAP patients demonstrated dysplasia compared to 1/18 (6%) of the sporadic FGPs ($p=0.01$).

Proliferative activity

All FGPs exhibited MIB-1 positive nuclei in epithelial cells located in the neck stem cell region. In all FGPs, both sporadic and syndromic, MIB-1 positivity was also seen in the epithelium lining the dilated cysts and in the glands adjacent to the cysts. There was no difference in proliferative index in the

dilated cysts between syndromic and sporadic FGPs without dysplasia: $2.7 \pm 0.3 \%$ vs. $4.7 \pm 0.9 \%$ (mean \pm SEM, $p=0.25$). In neck stem cell regions in FGPs without dysplasia, there was no difference in proliferative index between syndromic and sporadic FGPs: $5.4 \pm 1.8 \%$ vs. $4.5 \pm 0.9 \%$ ($p=0.41$). Within FGPs containing dysplasia, the proliferative index in the neck stem cell region was higher in dysplastic areas than in non-dysplastic areas: $16.4 \pm 4.8 \%$ vs $5.5 \pm 1.6 \%$ ($p=0.04$, figure 2). In the five FGPs with dysplasia, the proliferative index was also higher than in the 22 FGPs without dysplasia when assessed in cells lining the cysts: $8.7 \pm 2.8 \%$ vs $4.3 \pm 0.9 \%$ ($p=0.02$). There were no differences in proliferative indices between non-dysplastic areas of dysplastic FGPs and FGPs without dysplasia ($p=0.41$).

Apoptosis

Apoptotic cells were seen in all FGPs. The distribution of M30 positive cells throughout the FGP was variable with no clear pattern. The mean number of M30 positive cells was 5.0 ± 0.8 per 10 HPFs.

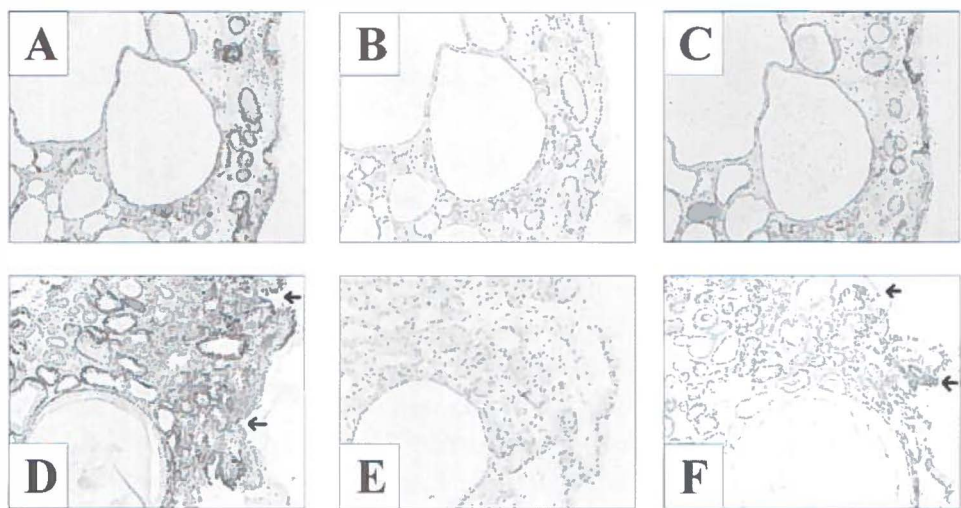


Figure 2. Example of a fundic gland polyp without dysplasia (A-C) and a fundic gland polyp with low-grade dysplasia (D-F). Serial hematoxylin-eosin (HE) staining (A, D) and immunohistochemical staining of MIB-1 (B, E) and β -catenin (C, F). In the FGP without dysplasia, relatively low MIB-1 positivity and membranous β -catenin staining is seen whereas a FGP with dysplasia (arrows) shows high proliferative activity and nuclear β -catenin staining. See appendix 4, page 188 for colour figure.

No difference was found in the mean number of apoptotic cells between syndromic and sporadic FGPs without dysplasia: 5.6 ± 1.0 vs 4.7 ± 1.2 ($p=0.97$). In the FGPs containing dysplasia the mean number of M30 positive cells was higher in the dysplastic areas than in the non-dysplastic areas 10.0 ± 3.1 vs 3.8 ± 0.9 ($p=0.04$). The number of M30 positive cells was comparable between non-dysplastic areas of dysplastic FGPs and non-dysplastic FGPs ($p=0.82$).

β -catenin expression

All FGPs stained positive for β -catenin. Nuclear accumulation of β -catenin was observed in only three FGPs. Two were syndromic FGPs, one with low, and one with high-grade dysplasia. The other one was the sporadic FGP with low-grade dysplasia. Nuclear β -catenin staining co-localised with dysplastic areas, with membranous staining in adjacent non-dysplastic areas and in the dilated cysts (figure 2). All other FGPs showed membranous staining. Nuclear staining was seen more often in FGPs with dysplasia (3/5) than in FGPs without dysplasia (0/22, $p<0.01$). Staining results for proliferative activity, apoptosis and β -catenin expression in FGPs with dysplasia are summarised in table 1.

Table 1: Results for proliferative activity, number of apoptotic cells and β -catenin staining, in the five FGPs containing dysplasia. Proliferative activity was assessed in the neck stem cell regions and expressed as proliferative index. Data are expressed comparing non-dysplastic areas (-) to dysplastic (+) areas in the same FGP.

Cases	Proliferation (%)		Apoptosis (per 10 HPF)		Nuclear β -catenin	
	-	+	-	+	-	+
1	4.0	33.3	3	5	Absent	Present
2*	2.4	4.6	5	10	Absent	Present
3	9.0	16.0	1	2	Absent	Absent
4	9.5	18.3	4	13	Absent	Absent
5	2.6	10.0	6	20	Absent	Present

* sporadic FGP

P53 expression

All FGPs, both syndromic and sporadic, displayed weak nuclear staining. Overexpression of p53 was not observed. There was no difference in p53 expression between dysplastic areas and non-dysplastic areas.

Discussion

Traditionally, FGPs have been regarded as benign lesions with no potential for malignant transformation. However, reports of FGPs harbouring dysplastic foci or even gastric adenocarcinoma, particularly when associated with FAP or AFAP, have raised new interest in the pathology of FGPs^{33,34}. In recent years, evidence has emerged for dysregulation of the Wnt-APC- β -catenin pathway in the development of FGPs. FAP associated FGPs are linked with second hit *APC* gene alterations^{23,35} whereas sporadic FGPs arise through mutations in the *β -catenin* gene^{24,36}. It has been suggested that *β -catenin* mutations are not equivalent to *APC* mutations in their functional biological consequences²⁴, as it has been firmly established that dysplasia develops preferentially in syndromic FGPs²⁷.

In this study in a series of syndromic and sporadic FGPs, including a subset of FGPs with dysplasia, differences in possible functional consequences of activation of the Wnt-APC- β -catenin pathway were examined. We found that syndromic and sporadic FGPs did not differ with respect to proliferative activity, degree of apoptosis, β -catenin or p53 expression. However, in the subset of FGPs with dysplasia, areas with dysplasia showed higher rates of cell turnover and more often nuclear β -catenin staining compared to areas without dysplasia.

Studies on proliferative activity in FGPs are limited but our data are largely in accordance with these studies. Others did not find differences in proliferative activity between syndromic and sporadic FGPs either^{3,13}. However, these studies were performed in non-dysplastic FGPs whereas the present study included dysplastic FGPs. We observed no difference in the degree of apoptosis between syndromic and sporadic FGPs. To the best of our knowledge, apoptosis has not been studied before in FGPs. Interestingly, it has been found that the degree of apoptosis was higher in hyperplastic gastric polyps with dysplasia than in those without dysplasia³¹, in line with our finding of higher apoptotic cell counts in dysplastic areas than in non-dysplastic areas. The higher degree of apoptotic cell death in dysplastic areas of the FGP may be linked to DNA damage in these cells or might be a direct consequence of the increased proliferative activity, leading to lack of nutrients, competition for growth factors, or oxygen starvation³⁷. Apoptosis can be initiated by wild-type p53 in response to DNA damage³². The absence of overexpression of p53 in

all FGPs suggests the presence of wild-type p53 that may be involved in the regulation of apoptosis in FGPs.

Data on β -catenin expression in FGPs are limited. Our results are in accordance with Abraham *et al* who described membranous and cytoplasmic β -catenin staining, but no nuclear staining, in non-dysplastic sporadic FGPs²⁴. Nuclear staining in FGPs, co-localising with dysplasia, has not been described previously. Interestingly, in both gastric and colorectal carcinogenesis translocation of β -catenin to the nucleus seems to be associated with tumour progression^{30,38}.

In our FGPs containing dysplasia, the dysplastic areas showed increased proliferative activity, higher degree of apoptosis and in most cases also nuclear β -catenin staining, indicating activation of the Wnt-APC- β -catenin pathway in these areas. The finding of higher cell turnover rates in dysplasia is not unexpected but has not been demonstrated before in FGPs. The non-dysplastic areas of the dysplastic FGPs did not have higher cell turnover rates than the non-dysplastic FGPs. This indicates that dysplasia in FGPs does not develop on a background of increased proliferative activity, as is generally found to be the case in colorectal adenomatous polyps^{39,40}. The finding of dysplasia in only a subset of FGPs suggests that additional genetic or functional alterations, besides mutations in the *APC* gene are needed to cause dysplasia. This is supported by a study in which no difference was found in the frequency of somatic *APC* mutations in syndromic FGPs with or without dysplasia²³. Our results of nuclear β -catenin staining in only a subset of dysplastic FGPs suggests that the development of dysplasia precedes nuclear translocation of β -catenin. Alternatively, it may be possible that dysplasia does develop after nuclear translocation of β -catenin but that immunohistochemistry is not sensitive enough to detect nuclear β -catenin in all dysplastic FGPs. It is also possible that the development of dysplasia in FGPs is mediated by the *APC* mutation independently of the Wnt-APC- β -catenin pathway. The *APC* protein also plays a role in cell-cycle control, migration and differentiation, each of which, when dysregulated, could contribute to development of dysplasia²⁵. Wei *et al* suggest that the alterations in the tumour suppressor *tuberin* (*TSC2*) in FGPs may block nuclear translocation of mutant β -catenin, leading to a benign cellular proliferation rather than a malignant transformation⁴¹.

There have been several reports of FGPs occurring in association with proton pump inhibitors (PPI) therapy, although this was not confirmed in a recent large

case-control study⁴²⁻⁴⁵. Some have found specific histologic differences between FGPs associated with PPI use and those not associated with PPI use, but others have not^{42,46,47}. In our study, 1 of 6 FAP patients and 8 of 18 sporadic patients had received prior therapy with PPIs. The use of PPIs in our population was not associated with the presence of dysplasia or other histologic characteristics of FGPs. Given the current knowledge of the genetic background of FGPs, evidence for a possible promoting role of PPIs in the pathogenesis of FGPs might come from *in vitro* studies that show activation of the Wnt pathway upon PPI administration. However, no such studies have yet been performed.

It is important to realise that the risk associated with FGPs containing dysplasia to develop into gastric carcinoma has not yet been determined. Whether the finding of severe dysplasia in a FGP is an indication for surgical intervention remains to be established. We recommend that all FGPs, which are large or look suspicious at endoscopy, are examined histologically to exclude the presence of dysplasia, particularly in patients with FAP or AFAP. Recently, it was suggested that epithelial gastric polyps larger than five mm should always be removed, however FAP patients and FGPs were not included in this study⁴⁸. If dysplasia is found in FGPs from a patient not previously diagnosed with FAP or AFAP, a lower gastrointestinal tract examination is indicated to exclude the presence of FAP or AFAP. Additional immunohistochemical staining of FGPs for β -catenin, proliferative activity and apoptosis may be helpful in confirming the presence of dysplasia.

In conclusion, syndromic and sporadic FGPs without dysplasia did not differ with respect to proliferative activity, degree of apoptosis, β -catenin or p53 expression, providing no support for the hypothesis that *APC* and *β -catenin* gene mutations have different functional consequences in FGPs. The frequent finding of dysplasia in syndromic FGPs and the association of dysplasia with relatively high cell turnover rates and nuclear expression of β -catenin suggest that the Wnt-APC- β -catenin pathway is activated in dysplasia. However, since not all syndromic FGPs are dysplastic, it seems that other genes in addition to the *APC* gene are involved in the activation of this pathway in dysplastic lesions in FGPs.

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Chapter 8

Increased risk of fundic gland polyps during long term proton pump inhibitor therapy

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Abstract

Objectives: To determine whether FGP development is associated with the use of proton-pump inhibitors (PPIs) and which mechanisms are involved.

Methods: In this case-control study, PPI use and its duration and the presence of FGPs were assessed in consecutive patients undergoing oesophagogastroduodenoscopy. Biopsies, from FGPs and normal gastric mucosa were prepared for routine histology. Dysplasia was graded as negative, low or high grade. Prevalence of parietal cell hyperplasia (PCH) and parietal cell protrusions (PCP) and the proportional cystic area were assessed.

Results: Data were obtained from 599 patients, of whom 322 used PPIs. Histologically confirmed FGPs were identified in 107 patients. Long-term PPI use was associated with an increased risk of FGPs (1 – 4.9 years use: OR 2.2, 95% CI 1.3-3.8 and 5 or more years use: OR 3.8, 95% CI 2.2-6.7) while short-term PPI therapy (< 1 year) was not (OR 1.0, 95% CI 0.5 - 1.8). Low-grade dysplasia was found in 1/107 FGPs. FGPs from patients with long-term PPI use were characterized by a larger proportional cystic area and a higher frequency of PCH and PCP. In the additional 33 FAP patients evaluated, the risk of FGPs and the risk of dysplastic FGPs were not increased with PPI use.

Conclusions: Long-term use of PPIs is associated with an up to 4-fold increase in the risk of FGPs. The risk of dysplasia in these polyps is negligible. Etiologically, these polyps seem to form as a consequence of parietal cell hyperplasia and parietal cell protrusions resulting from acid suppressive therapy.

Introduction

Fundic gland polyps (FGPs) are the most common gastric polyps ¹. They are found in up to 1.9% of the general population (known as sporadic FGPs) and in up to 84% of patients with familial adenomatous polyposis (FAP), where they are known as syndromic FGPs ²⁻⁴. FGPs are characterized histologically by distorted glandular architecture consisting of microcysts, mostly lined with chief and parietal cells ⁵. Interestingly, FGPs occur almost exclusively in patients without *H. pylori* infection ⁴. FGPs have always been regarded as benign lesions, with at most low-grade dysplasia (intraepithelial neoplasia). There have, however, been case-reports of FGPs harbouring severe dysplasia or even gastric adenocarcinoma, particularly when associated with FAP, but also in one sporadic case ⁶.

The potential association of FGPs with the use of proton pump inhibitors (PPIs) has been a topic of debate since these drugs were first prescribed ⁷. PPIs, which are currently widely used to inhibit gastric acid secretion, bind irreversibly to the hydrogen/potassium pump, leading to inhibition of acid secretion by the parietal cells. In 1992 Graham *et al* reported three cases of FGPs which developed after one year of treatment with the PPI omeprazole ⁸. Since this initial observation there have been numerous case reports and small series describing the occurrence of FGPs in association with long-term use of PPIs ⁹⁻¹⁴. In several of these cases regression of FGPs after cessation of PPI therapy was reported ^{9,13}. Two studies in larger series of patients have shown conflicting results regarding the prevalence of FGPs in patients on long-term PPI therapy as compared to a control group (table 1) ^{15,16}. Another four studies, published as abstract or letters, but never as full paper, indicate that there could be an association between FGPs and PPI use (table 1) ¹⁷⁻²⁰.

A case-control study in FAP patients suggested that the risk of dysplasia in FGPs increases during PPI use ²¹. Thus, although PPIs are widely regarded as safe, the discussion whether the association between FGPs and PPI use exists continues. It has even been suggested that routine endoscopies should be performed in patients on long-term PPI therapy to monitor FGP development ^{11,22} and the question whether there is a contra-indication for long-term PPI therapy in FAP patients remains unanswered. To settle this issue, it is necessary to assess the prevalence of FGPs in patients on long-term PPIs in comparison with patients not using PPIs. The relatively low prevalence of FGPs makes a case-control study most feasible ²³.

Table 1: Summary of case-control studies regarding the association between PPIs and FGPs

Author	Study type	PPI use			No PPI use		P
		N	Months	FGPs %	N	FGPs %	
Choudhry ^{15 c}	Retrospective	231	0-98	5	2072	0.3	< 0.001 ^b
Vieth ^{16 c}	Retrospective	2251	> 1	5	28,096	5	NS
Collins ^{18 d}	Retrospective	54	12-60	35	54*	0*	< 0.001 ^b
Graham ^{17 d}	Prospective	11	12	36	295	3	< 0.001 ^b
Mogadam ^{19 d}	Prospective	27	18-48	26	133	2	< 0.001 ^b
Reekmans ^{20 d}	Prospective	115	Long	9	2866	1	< 0.0001 ^a

*Baseline values for same patients; ^aFisher's exact test; ^bChi-squared test; ^cReported as full paper; ^dOnly reported as abstract or letter; N: number of patients; NS: not significant

The aims of this case-control study were to determine whether FGP development is related to PPI use. We hypothesized that longer PPI use is associated with a higher prevalence of FGPs and that the acid suppression is directly involved in the development of the FGPs. We collected data regarding PPI use in FAP and other patients before undergoing diagnostic oesophagogastroduodenoscopy and looked specifically for FGPs. When FGPs were found, biopsies were collected for histological verification and further investigation.

Materials and Methods

Patient characteristics

All patients undergoing diagnostic oesophagogastroduodenoscopy at the endoscopy department of the University Medical Center Groningen between November 2002 and March 2005 were asked to participate in the study and to complete a questionnaire regarding the use of PPIs. The type of PPI used, the dosage used and the length of use were recorded. The reported data were verified and completed using the hospital patient records where possible and, when necessary, missing information was obtained from the general practitioner. Intermittent PPI use was evaluated as no PPI use. PPI use was graded into three categories: no reported use, < 1 year PPI use and > 1 year PPI use. The category > 1 year PPI use was further divided into 1 - 4.9 years and 5 or more years PPI use. For each patient, the sex and age were also

recorded. A database containing information about FAP patients (n = 50) treated at the University Medical Center Groningen from 1970 to 2005 was available. In each patient, the diagnosis FAP had been established by the presence of hundreds of adenomatous polyps found at colonoscopy at an early age, histopathological examination and, where possible, APC gene mutation analysis. This database was used to identify patients with FAP in our study population. During the study period 33 FAP patients had one or more oesophagogastroduodenoscopies.

Collection of FGPs

All oesophagogastroduodenoscopies were performed by five experienced gastroenterologists who had been additionally instructed in the recognition of FGPs and the method of data collection. When FGPs were found, the following characteristics of the FGPs were registered: localization in corpus and/or fundus, number of FGPs, estimated maximal size and any unusual characteristics. At least two biopsies were taken from each of the following: the largest corpus FGP, the largest fundus FGP, normal looking antrum mucosa and normal looking corpus mucosa. The biopsies were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) for routine histology.

Histological Examination

The H&E slides were retrieved from the files of the Department of Pathology, University Medical Center Groningen. The histological classification of FGPs was revised by one pathologist (JW). The diagnosis FGP was confirmed when histological examination of the endoscopically identified polyp revealed fundic mucosa with cystically dilated glands as defined by Lee *et al*²⁴. Criteria for the diagnosis of dysplasia (intra epithelial neoplasia) included nuclear enlargement or pleomorphism, stratification, and hyperchromatism²⁵. Dysplasia was graded as negative, low grade or high grade, according to Wu *et al*²⁶. In the antral and corpus biopsies, the presence of *H. pylori* infection was noted for each patient using both H&E and Giemsa staining. In FGPs and biopsies of normal looking corpus mucosa from 15 patients from each PPI-use group additional histological analysis was performed. The proportional cystic area of each polyp was measured using photographs. Firstly, the total area of the FGP tissue segment was measured followed by the total area of the cysts within the FGP to determine the area of cysts as a percentage of the total area of the polyp (Image-pro plus, version 4.6, MediaCybernetics, Silver Spring, MD, USA). The presence of parietal cell hyperplasia (PCH) was determined by estimating the percentage of parietal cells in the total cell population of the FGP tissue

segment semi-quantitatively as absent (0%), < 50% parietal cells and > 50 % parietal cells. Parietal cell protrusions (PCP) were defined as prominent intra-luminal protrusions of parietal cell cytoplasm resulting in a serrated glandular lumen and quantified as absent, moderately present (< 50% of cells) or extensively present (> 50% of cells) in the FGP ²⁷.

Statistical analysis

SPSS for Windows software (SPSS Inc., Chicago, IL,USA) was used for all statistical analyses. Group sample sizes of n=300 PPI users and n=300 non-PPI users achieve 81% power to detect a difference of 8%, assuming the proportion of FGPs in the group non-PPI users is 10% (alfa = 5%, tested 2-sided). The odds ratios and 95% confidence intervals (CI) for presence of FGPs during PPI treatment were calculated. Differences between patient groups were tested using the Mann-Whitney U test and the chi-square test. Multivariate logistic regression analysis was used to determine influence of both age and PPI use on the prevalence of FGPs. P values of < 0.05 were considered significant.

Results

Patient characteristics

Table 2 summarizes the characteristics of patients described in this study.

Table 2: Patient characteristics

	Non-FAP patients	Patients with FAP
N	599	33
Male/female %	42 / 58	46 / 54
Mean age (range)	53 (17-88)	44 (15-92)
PPI use % (N)		
- None	46 (277)	82 (27)
- < 1 year	27 (162)	6 (2)
- > 1 year	27 (160)	12 (4)
FGP % (N)		
- Absent	82 (492)	42 (14)
- Present	18 (107)	58 (19)

Patients with FGPs were older than patients without FGPs (60 years ± 12 vs. 52 years ± 16 respectively, mean ± SD, P < 0.001). As shown in table 2, the

mean age of non-FAP patients on long term (> 1 year) PPI-therapy (58 ± 13 , mean \pm SD:) was higher than that of patients not using PPIs (51 ± 17 , mean \pm SD, $P < 0.001$) and patients on short-term (< 1 year) PPI therapy (53 ± 15 , mean \pm SD, $P = 0.001$). 33 of the 50 FAP patients underwent oesophagogastroduodenoscopy during the study period. As expected the mean age of these 33 patients was lower than that of non-FAP patients (44 ± 16 vs. 53 ± 16 , mean \pm SD, $P < 0.001$). None of the patients with FGPs had *H. pylori* or signs of gastritis.

FGPs are associated with long-term PPI use

The prevalence of FGPs in non-FAP patients without PPI therapy and on short- and long-term PPI therapy is shown in table 3.

Table 3: FGPs in patients with no, < 1 year and > 1 year of PPI use

	No PPI use	< 1 year PPI	> 1 year PPI
N	277	162	160
Male % (N)	38 (105)	46 (75)	44 (70)
Age, mean (range)	51 (17-88)	53 (21-83)	58 (22-86)
FGPs % (N)	12 (32)	11 (18)	36 (57)

PPI therapy was associated with an increased risk of FGPs (OR 2.3, 95% CI 1.5 - 3.6). Subgroup analysis showed that short-term PPI therapy (< 1 year) was not associated with an increased risk of FGPs (OR 1.0, 95% CI 0.5 - 1.8). Long-term PPI use (> 1 year) was associated with an increased risk of FGPs (OR 2.8, 95% CI 1.8 - 4.5). Within the group of long-term PPI users the risk was higher in patients with five or more years of PPI therapy (OR 3.8, 95% CI 2.2 - 6.7) than for patients with 1 - 4.9 years of PPI therapy (OR 2.2, 95% CI 1.3-3.8). These results are summarized in figure 1.

Multivariate logistic regression analysis showed that both age and PPI use were independent predictors of the presence of FGPs ($P < 0.001$ for both). There was no difference in FGP risk in patients treated with omeprazole as compared to other PPIs, indicating that the effect is a class effect and not related specifically to omeprazole.

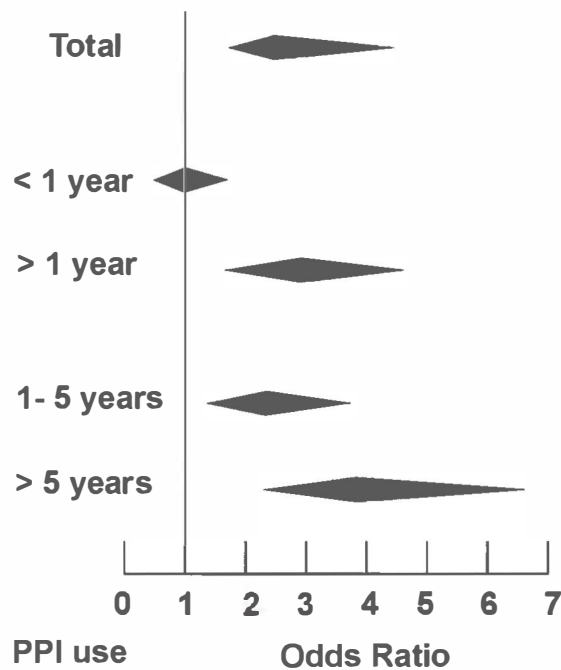


Figure 1: Odds ratios (with corresponding 95% confidence intervals) for fundic gland polyps (FGPs) in patients using proton pump inhibitors (PPIs). "Total" shows the risk of all PPI users vs. patients who had never used PPIs. < 1 year, > 1 year, 1-5 years and > 5 years show the risk of FGPs in patients who had used PPIs for these time periods as compared to patients who had never used PPIs.

Endoscopic and histological characteristics of FGPs

There were no differences in the localization, size or number of FGPs found in patients without PPI use, patients with short-term (< 1 year) PPI use and patients with long-term (>1 year) PPI use (table 4). In the series of non-FAP FGPs dysplasia was very rare. In 1/107 FGPs there was a focus of low-grade dysplasia. In the remaining non-FAP FGPs no dysplasia was found. Dysplasia was much more common in FAP-associated FGPs: 46 % of these FGPs were dysplastic, 8 % had high grade dysplasia and 38 % low-grade dysplasia.

Table 4: Characteristics of non-FAP FGPs

	No PPI use	Short-term PPI use	Long-term PPI use
Number of patients	32	18	57
Localisation (%)			
- corpus	44	33	30
- fundus	31	33	30
- both	25	34	40
Number (%)			
- 1 – 3	41	28	35
- 4 – 10	37	44	42
- > 10	22	28	23
Size (%)			
- "small" undefined	38	6	33
- 1 - 4 mm	28	22	17
- 5 – 9 mm	19	22	18
- > 10 mm	6	22	11
- unknown	9	28	21

In FGPs, PCH and PCP were more frequent in both the FGPs and the biopsies of normal gastric corpus mucosa from patients with long term (> 1 year) PPI use compared to patients with no reported PPI use (figures 2 and 3). The proportion of the FGP consisting of cysts was higher in the FGPs from patients with long-term (> 1 year) PPI use ($15\% \pm 2\%$, mean \pm SEM) than in FGPs from patients without reported PPI use ($9\% \pm 2\%$, mean \pm SEM, $p = 0.035$) (figure 3).

PPI use is not related to FGP development in FAP patients

Three FAP patients with and three FAP patients without FGPs were on PPI therapy. Two FAP patients had a large adenomatous polyp, both with low-grade dysplasia, in a bed of FGPs. Endoscopy three years earlier in both patients had shown FGPs, but no adenomas, in one patient and no abnormalities in the other patient. The first of these two patients used PPIs, but only for 1 month. There was also one FAP patient with high grade dysplasia in a FGP, but this patient was not on PPI therapy either. Of the remaining patients ($n = 16$), 30% had low-grade dysplasia in the FGPs, with only one of these patients using PPIs.

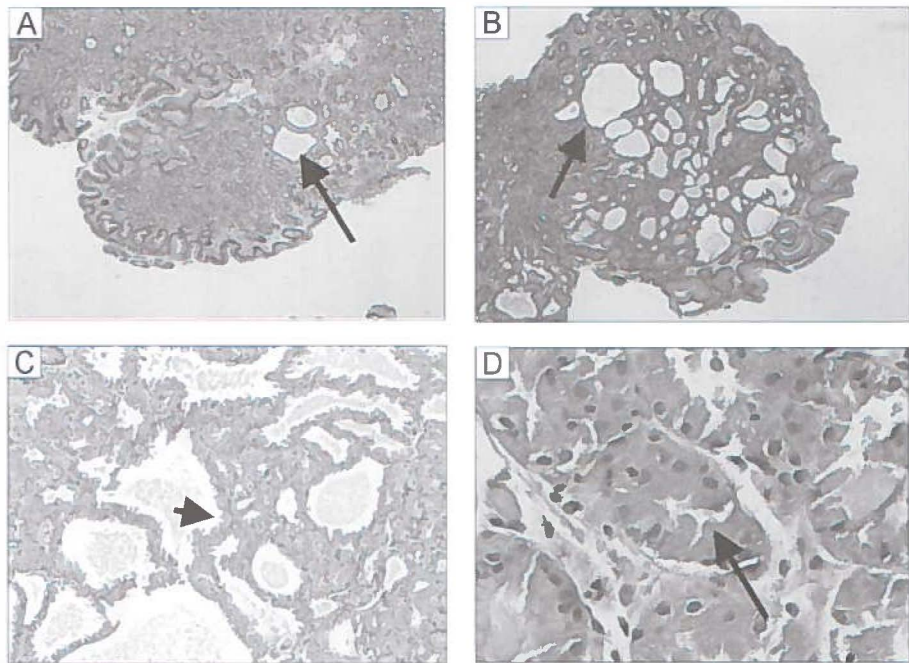


Figure 2: H&E stained tissue sections of fundic gland polyps (FGPs). (A) FGP from a patient who had never used proton pump inhibitors (PPIs) showing a few small cysts (arrow), original magnification 40x. (B) FGP from a patient with more than 1 year of PPI use showing a large number of cysts (arrow), original magnification 40x. (C) FGP from a patient with more than 1 year of PPI use showing parietal cell hyperplasia and parietal cell protrusions (arrow), original magnification 100x. (D) FGP from a patient with more than 1 year of PPI use showing parietal cell hyperplasia and parietal cell protrusions (arrow), original magnification 400x. See appendix 4, page 189 for colour figure.

Discussion

In this study we show that long-term use of PPIs is associated with a 4-fold increase in the risk of developing FGPs. Furthermore, we show that the risk of FGPs increases with longer PPI use. Microscopically, FGPs from patients treated with PPIs were characterized by more and/or larger cysts and more frequent PCH and PCP in both the FGPs and the normal looking corpus mucosa compared to FGPs and normal mucosa from patients without PPI use. This data supports an aetiology related to acid suppressive therapy.

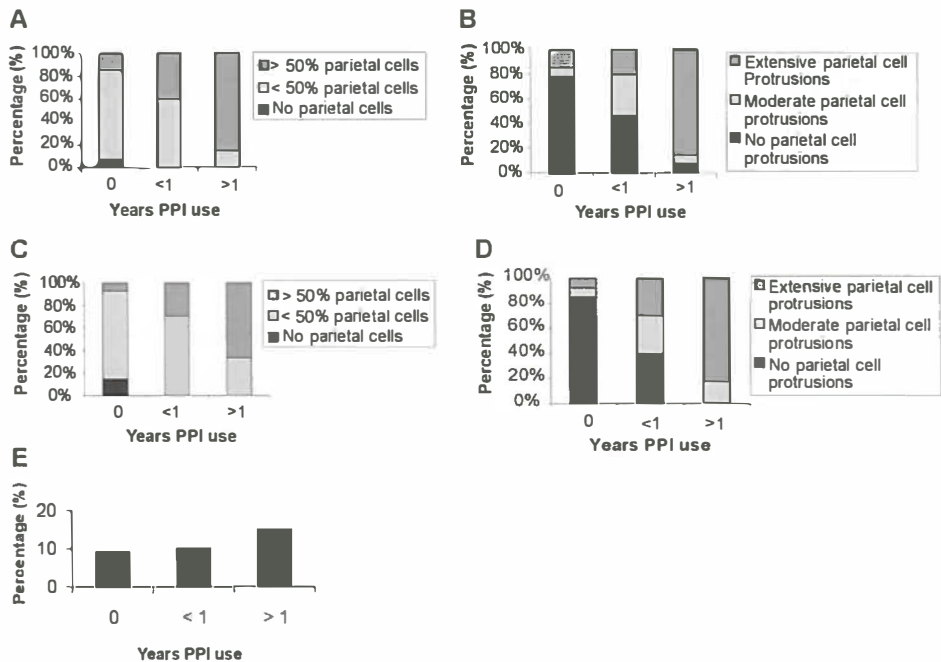


Figure 3: Parietal cell hyperplasia in FGPs (A) and normal looking gastric corpus mucosa (C) expressed depending on length of PPI use. Differences between groups were significant ($p < 0.01$). Parietal cell protrusions in FGPs (B) and normal looking gastric corpus mucosa (D) expressed depending on duration of PPI use. Differences between groups were significant ($p < 0.01$). (E) Mean percentage of surface area made up of cysts in FGPs from patients without PPI use and from those with <1 or >1 year of PPI use. Mean percentage of cysts is higher in patients on >1 year PPI therapy(*) compared to the mean percentage in FGPs from patients with no PPI use ($p = 0.035$).

Although numerous case reports and small series have described FGPs developing during long-term PPI use, studies, designed to prove a firm association, have shown conflicting results. The incidence of FGPs after (long-term) PPI therapy has been reported to be between 1 and 36%¹⁵⁻²⁰. In the largest retrospective study, with over 30,000 patients, Vieth *et al* showed no difference in the prevalence of FGPs between controls and patients after at least 4 weeks of PPI therapy¹⁶. This is not surprising considering that in our study and all other positive studies an association was only found after at least 12 months of PPI therapy. In accordance with this, in the retrospective study by Choudry *et al*, a higher risk of FGP development was found after a mean of 37

months of PPI therapy¹⁶. Four other studies which have only been reported in abstract form or as a letter have all shown an increased risk of FGP development after at least 12 months of PPI therapy¹⁷⁻²⁰. Our study is the first to demonstrate that the risk of FGPs continues to increase with longer PPI use. Data on length and frequency of PPI use was based on patient recall, so this may have introduced a bias. However, the data for the majority of the patients could be confirmed using hospital records and general practitioner records, thereby reducing the chance that this inaccuracy significantly influenced the results.

Dysregulation of the Wnt-APC- β -catenin pathway, as well as CpG island methylation and cellular distribution of tuberin and the glucocorticoid receptor, have been shown to play a role in the development of both non-FAP and FAP-associated FGPs, independently of PPI use^{2,28-31}. The mechanisms through which FGPs develop during PPI therapy are, however, largely unknown. The development of hyperplasia and protrusions of the parietal cells (PCH and PCPs) is thought to be an initial step³². PCPs are defined as hypertrophic parietal cells showing tongue-like protrusions of the apical membrane into the lumen of corpus glands. Cats *et al* found that the prevalence of PCP increased during PPI therapy³³. The largest increase was seen in the first 3 months of PPI use and this was related to a rise in serum gastrin levels³³. It is thought that the morphological changes leading to PCP occur because the secretory canaliculi are filled with hydrochloric acid, additionally stimulated by higher levels of gastrin, but active secretion of the acid is inhibited by the PPI therapy³³. A second step in the formation of FGPs is thought to be fundic gland cyst (FGC) formation³³. FGCs are intra-mucosal cysts which are believed to form from glands which are dilated due to increased intra-glandular pressure. This increase in pressure could be caused by increased resistance to outflow from the gland due to blockage of the isthmus by PCPs³⁴. Cats *et al* described FGC prevalence to increase from 8% at baseline to 35% after 12 months of PPI therapy³³. FGCs can present endoscopically as FGPs when they become larger.

Interestingly, FGPs develop almost exclusively in *H. pylori* negative patients and FGCs also develop more often in *H. pylori* negative patients³⁵. The absence of *H. pylori* infection in our patients with FGPs is in accordance with these previous observations. It has been suggested that enzymatic degradation of gastric mucus by *H. pylori* protease may facilitate the glandular outflow and thus protect against retention and cystic dilation³³. This does not take place in

H. pylori negative patients thus increasing the risk of FGP development. In summary, present data indicate that PCPs develop in the majority of patients during the first months of PPI therapy in association with an increase in serum gastrin level. During the first year of PPI therapy a subgroup of patients without *H. pylori* infection develop fundic gland cysts which are not visible endoscopically but can be identified in biopsies of normal looking gastric mucosa³³. As also described in the present study, an increased prevalence of macroscopic FGPs can be seen after at least 12 months of PPI therapy¹⁷⁻²⁰. These data suggest that the aetiology of PPI-associated FGPs differs from that of FGPs which develop without PPI use.

Based on our data, the development of FGPs and/or dysplasia in FGPs in FAP patients does not seem to be related to PPI therapy. Attard *et al.* found dysplasia in FGPs in two of two paediatric FAP patients on long term PPI therapy as compared to three of seven paediatric FAP patients not using PPIs²¹. We did not find this association in our adult population and it has not been reported elsewhere suggesting that the observation by Attard *et al* was based on chance or is specific for the paediatric population.

Although interesting from a mechanistic point of view, there seem to be few clinical implications of the association between long-term PPI therapy and FGP development since there is not an increased risk of cancer developing in these polyps. In conclusion, long-term use of proton pump inhibitors is associated with a 4-fold increase in the risk of developing fundic gland polyps. The risk of dysplasia in these polyps is not increased suggesting that additional endoscopic evaluation of these polyps is not indicated.

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Chapter 9

Summary, conclusions and future perspectives

Summary

Colorectal cancer is an important cause of cancer-related morbidity and mortality in the Western world ¹. Six percent of the general population develops colorectal cancer. Most colorectal cancers develop from precursor lesions known as adenomas. Although unhealthy diet and lifestyle are risk factors, clinical trials evaluating the effects of lifestyle improvements have generally failed to reduce the colorectal cancer development ². Screening of the general population through faecal occult blood testing, flexible sigmoidoscopy or colonoscopy are the most feasible, cost-effective means of reducing colorectal cancer mortality ³. Colorectal cancer is characterised by autonomous growth, invasion into surrounding tissue and metastasis, mainly to the regional lymph nodes, liver and lung. Although the majority of patients with colorectal cancer undergo resection with curative intent, many of these patients develop metastatic disease ⁴. In recent years, using novel chemotherapeutic regimes, there have been encouraging improvements both in the adjuvant setting and in the treatment of metastatic disease ^{5,6}.

Chemotherapy for colorectal cancer is hampered by intrinsic and acquired resistance to chemotherapy. Colorectal carcinogenesis is hallmarked by a relative resistance to apoptosis and therefore there is much interest in understanding (dysregulation of) apoptotic pathways and in the development of targeted apoptosis-inducing drugs. Apoptosis results from the regulated activation of a death program that is encoded in the genome. Interestingly, besides being activated by genetic defects and cell damage due to radiation or toxic agents, apoptosis can also be induced by ligand activation of cell-surface death receptors. Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is a member of the TNF family which also includes TNF and Fas Ligand. It induces apoptosis in a wide variety of cancer cell lines including colon cancer cell lines and colon cancer xenografts while leaving normal tissue unharmed ⁷. TRAIL induces apoptosis by binding to its pro-apoptotic receptors DR4 and DR5 leading to the activation of proteins which can directly or indirectly initiate apoptosis. TRAIL-based therapies are currently in early stage clinical testing. The aims of the first part of this thesis were to determine the potential of TRAIL-induced apoptosis in adenomas, to investigate TRAIL-receptor regulation in colorectal neoplasms and to investigate the effects of the combination of TRAIL with non-steroidal anti-inflammatory drugs (NSAIDs). The aims of the second part of this thesis were to study the changes involved

in the transition from normal to dysplastic tissue in fundic gland polyps of the stomach.

Following a brief introduction and outline of this thesis in **chapter 1**, the potential of combinations of NSAIDs with agents targeting specific signalling pathways in the chemoprevention of colorectal cancer are reviewed in **chapter 2**. NSAIDs have repeatedly been shown to reduce the risk of colorectal cancer development in both retrospective studies and in randomised controlled trials. These drugs do not, however, offer complete protection against adenoma or carcinoma development. The risk of side-effects associated with long-term NSAID use outweigh the benefits of reduced cancer risk in almost all patient groups. By combining low-dose NSAIDs with agents which increase effectivity of chemoprevention by NSAIDs, this preventive treatment could potentially be extended to larger patient groups. This review discusses the literature regarding the mechanisms and the *in vitro* and *in vivo* effectivity of several drugs as well as the rationale for combining these drugs with NSAIDs. The first class of drugs discussed are the peroxisome proliferator-activated receptor γ (PPAR γ) ligands which are currently widely used in the treatment of diabetes mellitus. PPAR γ ligands reduce the growth rate of human colon cancer cells *in vitro*; alarmingly however, rodent models have shown contradictory results regarding the prevention of adenomas, with treatment increasing the rate of adenoma development in some models. These results need to be explained before clinical trials can be contemplated. Cholesterol synthesis inhibitors (statins) are frequently prescribed in patients with increased cholesterol levels to reduce the risk of cardiovascular disease. Like the PPAR γ ligands, long-term statin use is associated with very few side-effects. Statins have been shown to reduce colon cancer cell growth *in vitro* and both rodent models and retrospective studies indicate a chemopreventive effect for colorectal cancer of these drugs. The efficacy of epidermal growth factor (EGF) signalling inhibition in colorectal cancer has been demonstrated *in vitro* and *in vivo* in preclinical and clinical studies. This recently resulted in the registration of cetuximab, a monoclonal antibody against the EGF receptor (EGFR), for use in irinotecan refractory colorectal cancer. Finally, TNF-related apoptosis inducing ligand (TRAIL) induces apoptosis in human colon cancer cells, but not in normal cells and the first clinical trials with TRAIL-based drugs are currently ongoing. EGF signalling inhibitors and TRAIL-based drugs are both potentially active in adenomas. However, there is still a long way to go before there is enough clinical experience with these novel drugs to contemplate clinical studies in the preventive setting. All these drugs have all been shown to interact with

NSAIDs leading to enhanced effects in colorectal cancer cells and/or in rodent models. In conclusion, these combinational regimens are an interesting future strategy for the chemoprevention of colorectal cancer.

To further explore the combination of NSAIDs with TRAIL, as described in chapter 2, it is necessary to know whether TRAIL, which is effective in many types of (colon) cancer cells but not in normal cells, is also effective in premalignant adenomas. Previous studies have shown that the pro-apoptotic TRAIL-receptors are expressed at a higher level in adenomas than in normal colorectal tissue. In **chapter 3** it was investigated whether it was possible to induce apoptosis in human adenoma cell lines and human adenomas using rhTRAIL. To study this, two human adenoma cell lines were exposed to rhTRAIL. This resulted in up to 55% apoptosis which was accompanied by caspase-3 and caspase-8 activation and could be inhibited by a pan-caspase inhibitor indicating that the apoptosis was caspase dependent. There is much discussion regarding the validity of colon adenoma cell lines as a model for human colorectal adenomas. Therefore, short-term explant cultures were established from freshly removed human adenomas (n=38) and biopsies of normal colon epithelium (n=15). RhTRAIL induced caspase-dependent apoptosis in adenomas with high-grade dysplasia (n=21) compared to paired untreated counterparts as determined in paraffin-embedded tissue using morphological criteria and staining for cleaved caspase-3. This apoptosis could also be inhibited using a pan-caspase inhibitor indicating the caspase dependent nature of the apoptosis. RhTRAIL did not induce apoptosis in adenomas with low-grade dysplasia (n=17) or in normal colon epithelium (n=15). This study provides evidence that both colorectal adenoma cell lines and adenomas with high-grade dysplasia are sensitive to rhTRAIL-induced apoptosis whereas normal colon epithelium is not. These results suggest that rhTRAIL could potentially be used in the treatment of adenomas with high-grade dysplasia. This is especially interesting in the treatment of endoscopically irresectable adenomas from patients with hereditary forms of colorectal cancer. If these adenomas could be reduced in size to make them resectable, a colectomy could be prevented or delayed.

The results described in chapter 3 demonstrate that the increased TRAIL-receptor expression in adenomas results in functionality of the TRAIL-mediated apoptosis pathway. In future, this pathway may be targeted in the treatment of colorectal adenomas and/or carcinomas and it is therefore important to understand why the pro-apoptotic TRAIL receptors are up-regulated in these tumours. This was investigated in **chapter 4**. One of the most important

pathways involved in colorectal cancer is the Wnt pathway. In colorectal cancer this pathway is usually activated by a mutation in the *adenomatous polyposis coli* (APC) gene. Due to this mutation the β -catenin protein cannot be properly degraded and accumulates in the cytoplasm, eventually being translocated to the nucleus of the cell. In the nucleus β -catenin binds to the T-cell factor (TCF) transcription factor and thus induces the transcription of genes associated with proliferation and apoptosis. It is tempting to hypothesise that this important pathway is also responsible for the increase in TRAIL-receptor expression in colorectal adenomas and carcinomas. To investigate whether there is an association between β -catenin and DR4/5, β -catenin was down-regulated using either β -catenin short-interfering-RNAs or an inducible RNA-interference construct in human colorectal adenoma and carcinoma cell lines. Down-regulation of β -catenin resulted in lower total cellular DR4 and DR5 protein levels. Similarly, cell membrane expression of DR4 and DR5 was reduced after down-regulation of β -catenin in colon cancer cell lines. β -catenin suppression not only decreased the levels of DR4 and DR5 but also decreased rhTRAIL sensitivity. To determine whether the described effects of β -catenin on DR4/5 expression are mediated through TCF-4, this transcription factor was down-regulated using inducible constructs. This β -catenin-independent down-regulation of TCF-4 signalling, however, did not affect TRAIL-receptor expression or rhTRAIL sensitivity. The association between β -catenin and DR4/5 was further investigated in human colorectal adenomas and carcinomas. Colorectal tumours with aberrant (cytoplasmic and nuclear) β -catenin expression had a higher percentage of immunohistochemical DR4 and DR5 staining per tumour than those with membranous β -catenin staining only. Furthermore, in serial slides aberrant β -catenin staining was shown to co-localise with both DR4 and DR5 expression in the majority of adenomas. In human colorectal carcinomas aberrant β -catenin expression was present in most cases and DR4/5 expression was largely homogenous. These results indicate that the gradual increase in TRAIL-receptor expression during colorectal carcinogenesis is at least partially mediated through increased β -catenin expression, but that this seems to be independent of TCF-4-signalling. An understanding of TRAIL-receptor regulation is important to be able to predict effectivity and potential drug interactions when rhTRAIL is combined with other agents.

As described in chapter 2 there are indications that NSAIDs and rhTRAIL may have synergistic effects. The question whether the NSAID aspirin can enhance rhTRAIL-induced apoptosis in colon cancer cells was investigated in **chapter 5**.

Although many colon cancer cell lines are sensitive to rhTRAIL, both intrinsic and acquired resistance to TRAIL have been described. In this study human colon cancer cell lines with varying sensitivity to rhTRAIL were used as well as a cell line which has been made TRAIL resistant and a cell line in which dominant negative (dn) expression of TCF can be induced using doxycyclin. Aspirin sensitized all cell lines to rhTRAIL-induced apoptosis in a dose-dependent manner. Addition of 10 mM aspirin led to a 2 – 9 fold increase in rhTRAIL-induced apoptosis measured using fluorescence microscopy. Increased apoptosis was accompanied by increased M30 staining and caspase-3 activation. Overexpression of dnTCF-4 completely blocked the sensitizing effect of aspirin on rhTRAIL induced apoptosis. These results show that aspirin sensitizes colon cancer cell lines with intrinsic or acquired TRAIL-resistance to rhTRAIL-induced apoptosis and this effect is mediated through Wnt signalling.

For the development and use of drugs which target specific pathways in (pre)malignant lesions it is important to understand more about pathways involved in the development of dysplasia and malignancy. One way of approaching this is by trying to understand why certain changes lead to dysplasia or malignancy in some cases but not in others. The final three chapters investigate so-called fundic gland polyps. These polyps are the most common gastric polyps and are found in up to 2% of the general population, occur in the fundus and corpus of the stomach and were originally thought to be completely benign⁸. However, in patients with FAP in whom these polyps are much more frequent, they can develop dysplasia and have been found to become malignant in some cases. In **chapter 6** we describe a case of sporadic fundic gland polyps (FGPs) containing high-grade dysplasia in a 68-year-old man. High-grade dysplasia has never been described in FGPs from a non-FAP patient, however, two colonoscopies in the previous six years had ruled out FAP in this patient. Additional immunohistochemical investigations showed nuclear staining of β -catenin and increased proliferation and apoptosis in the dysplastic areas of the FGPs indicating Wnt pathway activation. This case suggests that the malignant potential of FGPs is not limited to FAP-associated FGPs.

The differences in the potential for the development of dysplasia in FGPs in FAP and non-FAP patients make it interesting to study these gastric polyps and this is described in **chapter 7**. FAP-associated FGPs, like the colorectal adenomas and carcinomas in these patients, arise through mutations in the

APC gene. Non-FAP FGPs occur due to mutations in the *β-catenin* gene. Dysplasia in non-FAP FGPs, found less often than in FAP-associated or syndromic FGPs, is associated with *APC* rather than *β-catenin* mutations. These data suggested different functional consequences of *APC* and *β-catenin* mutations. To investigate this hypothesis, proliferative activity, degree of apoptosis, *β-catenin* expression and p53 expression in FAP and non-FAP FGPs were investigated using immunohistochemistry. We saw that both kinds of FGPs exhibited similar proliferative activity, degree of apoptosis, *β-catenin* and p53 expression when no dysplasia was present. As expected, dysplasia was observed more often in FAP-associated (4/9) than in non-FAP FGPs (1/18). Within the FGPs containing dysplasia, dysplastic areas showed abnormal nuclear *β-catenin* staining in 3/5 cases and higher rates of cell proliferation and apoptosis than non-dysplastic areas. Overexpression of p53 was not observed. The finding of similar rates of proliferation and apoptosis in FAP and non-FAP FGPs does not support the hypothesis that *APC* and *β-catenin* gene mutations have different functional consequences in FGPs. The association of dysplasia with relatively high cell turnover rates and nuclear expression of *β-catenin* indicates activation of the Wnt-*APC*-*β-catenin* pathway in dysplasia. The finding of dysplasia in some but not all syndromic FGPs suggests the involvement of other genes, in addition to the *APC* gene, in the development of dysplasia in FGPs.

The potential for the development of dysplasia in FGPs has raised some questions regarding their clinical consequences. FGPs are often found accidentally during an endoscopy since they generally do not cause gastrointestinal complaints themselves. This discussion is especially relevant in patients using proton pump inhibitors (PPIs) to reduce acid secretion in the stomach. Since the introduction of PPIs in the 1980s, there have been reports regarding a possible increased risk for FGP development during long-term PPI use. Two large studies addressing this question showed conflicting results. In the case-control study described in **chapter 8**, we assessed the prevalence and duration of PPI use and the prevalence of FGPs in a consecutive group of patients undergoing esophagogastroduodenoscopy. Data were obtained from 599 patients, of whom 322 were on PPI therapy and histologically confirmed FGPs were identified in 107 of these patients. Long-term PPI use was associated with an increased risk of FGPs (1 – 4.9 years use: OR 2.2, 95% CI 1.3-3.8 and 5 or more years use: OR 3.8, 95% CI 2.2-6.7) while short-term PPI therapy (< 1 year) was not (OR 1.0, 95% CI 0.5 - 1.8). Biopsies of the FGPs were studied and low-grade dysplasia was found in 1/107 FGPs. FGPs from

patients on long-term PPI use were found to have a larger proportional cystic area accompanied by hyperplasia and protrusions of the parietal cells indicating an aetiology related to the acid suppressive therapy. Therefore, although we provide evidence that the prevalence of FGPs is increased in patients on long-term PPI therapy, the risk of dysplasia in these polyps seems negligible and long term follow-up for dysplasia is not necessary.

Conclusions and Future Perspectives

Specific apoptosis induction is attractive in the treatment of colorectal cancer which is hallmarked by a relative resistance to apoptosis and limited responses to traditional chemotherapeutic drugs. The death-receptor ligand rhTRAIL can induce apoptosis in both colon adenoma and carcinoma cell lines as well as in short-term cultures of adenomas.

The potential of rhTRAIL-based therapies relies on the difference in TRAIL sensitivity between normal and tumour cells which is, at least partly, based on TRAIL-receptor expression. Colorectal adenomas and carcinomas express the pro-apoptotic death receptors DR4 and DR5. Intuitively, it is strange that colorectal tumours express receptors which render them susceptible to apoptosis induction by TRAIL. We have shown that overexpression of the β -catenin protein, which plays an important role in colorectal carcinogenesis, also plays a role in the upregulation of the TRAIL-receptors. Understanding of receptor regulation is important to determine which combinations of drugs could maximise receptor expression or functionality in tumours, but it will also eventually help to minimise or prevent unwanted side-effects. An alarming example of this was seen in patients with breast cancer. The use in such patients of trastuzumab, a monoclonal antibody targeting the EGF-receptor HER-2, in combination with anthracycline-based chemotherapy led to significant cardiotoxicity, possibly as a consequence of cardiac upregulation of the HER-2 receptor⁹. Current research focussing on mechanisms of TRAIL-receptor regulation, the roles of the different receptors in TRAIL-induced apoptosis and the affinities of the various receptors for native and modified forms of TRAIL is therefore very important^{10,11}.

Not all colon cancer cell lines are sensitive to TRAIL and in our ex-vivo model only adenomas with high grade dysplasia were TRAIL-sensitive. It is important to understand the reasons for differences in TRAIL-sensitivity, not only to

anticipate the effect of TRAIL in different situations, but also to find ways of increasing response to TRAIL. TRAIL sensitivity can be influenced by many factors including mutation status of p53, p21 and PTEN as well as the integrity of TRAIL pathway components^{12,11,13}. Current research points at important roles for the ratio between caspase-8 and FLIP, the clustering of receptors in lipid-rafts in the cell membrane and down-regulation of anti-apoptotic TRAIL-pathway proteins such as Bcl-2 and XIAP in determining sensitivity to rhTRAIL. *In vitro*, TRAIL-sensitivity can be enhanced not only with NSAIDs, but also with various traditional chemotherapeutic drugs and novel drugs such as proteasome inhibitors¹². Drugs which increase TRAIL-sensitivity mostly act on one or more of the above mentioned determinants of TRAIL-resistance although the exact mechanisms are currently unclear¹². The optimization of short-term culture models is important in this setting so that sensitivity to apoptosis induction by single drugs and drug combinations and the signalling pathways involved can also be studied in ex-vivo patient material. Future studies investigating the effects of rhTRAIL, alone and in combination with other drugs, in rodent models of sporadic and hereditary colorectal carcinogenesis are also important. These studies could pave the way for patient studies using TRAIL-based therapies in colorectal cancer.

Phase I/II studies with TRAIL-based therapies, both rhTRAIL and agonistic antibodies against the pro-apoptotic TRAIL receptors, are ongoing in patients with advanced cancer¹⁴. If the encouraging early results regarding toxicity profiles for rhTRAIL-based therapy continue, phase II studies exploring the effects of rhTRAIL on adenomas in patients with FAP, could become feasible next to phase II/III trials in patients with advanced cancer. An interesting application of short-term cultures of biopsies of colon adenomas or carcinomas is in the prediction of rhTRAIL sensitivity of the tumour before treatment commences. For clinical studies with rhTRAIL and other novel targeted anti-cancer drugs methods of patient selection and treatment evaluation are becoming increasingly important. Patient selection in early stage clinical trials may become necessary to optimize treatment efficacy since many biological parameters in both patients and tumours influence response to and toxicity of targeted drugs. In breast cancer the discovery of the estrogen and progesterone receptors, and more recently the HER-2 receptor, have already led to patient-tailored treatment and increased survival in this cancer type¹⁵. Although some prognostic in colorectal cancer have been identified, molecular markers which can predict the efficacy of chemotherapeutic regimes in colorectal cancer have not yet been discovered¹⁶. This indicates that the situation is dependent on a large combination of factors and may be extremely complex. For this reason, it

is essential to continue to study the mechanisms of carcinogenesis as well as the mechanisms associated with drug efficacy and resistance in colorectal cancer. This could eventually result in the use of molecular characteristics of the patient and the tumour to select patients likely to benefit from different treatment strategies.

The current opinion is that combined modality treatment including both traditional chemotherapeutic drugs and drugs targeting specific signalling pathways is the way forward in cancer therapy. After many years of gradually increasing knowledge regarding signalling pathways involved in carcinogenesis, two targeted drugs, monoclonal antibodies targeting the epidermal growth factor receptor 1 and vascular endothelial growth factor, respectively, have recently been registered for the treatment of metastatic colorectal cancer¹⁷. In the further development of combinations of (targeted) anti-cancer drugs, such as TRAIL, knowledge regarding the molecular mechanisms involved in carcinogenesis, drug-effectivity and drug-interaction are essential.

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Chapter 10

Summary in Dutch

Nederlandse samenvatting

Samenvatting

Darmkanker, of colorectaalcarcinoom, is een belangrijke oorzaak van kankergerelateerde ziekte en sterfte in de westerse wereld ¹. Zes procent van de algemene bevolking ontwikkelt, in de loop van het leven, darmkanker. Deze ziekte ontstaat meestal in goedaardige gezwellen van de darmwand, zogenaamde adenomateuze poliepen of adenomen. Een ongezonde leefwijze verhoogt de kans op colorectaalcarcinoom. Het bevorderen van een gezonde leefwijze heeft echter nog niet geresulteerd in minder darmkankerontwikkeling ². Bevolkingsonderzoek door middel van endoscopisch onderzoek van de dikke darm, of door het aantonen van bloed in de ontlasting, zijn wel haalbare en kosteneffectieve strategieën om sterfte aan darmkanker te verminderen ³. Het colorectaalcarcinoom kenmerkt zich door omgevingsonafhankelijke, ongecontroleerde celgroei, invasie in omliggende weefsels en uiteindelijk uitzaaiingen, met name naar (lokale) lymfeklieren, de lever en de longen. De meerderheid van de patiënten met darmkanker ondergaat een in opzet curatieve operatie, maar op langere termijn blijken veel patiënten toch uitzaaiingen (metastasen), te ontwikkelen ⁴. Gebruikmakend van nieuwe combinaties van chemotherapie is de laatste jaren vooruitgang geboekt in de behandeling van deze ziekte, zowel in de de adjuvante setting als in de gemetastaseerde setting ^{5,6}.

Verworven en intrinsieke resistentie voor chemotherapie zijn een groot probleem in de behandeling van gemetastaseerde darmkanker. Darmkankerontwikkeling wordt gekenmerkt door een relatieve resistentie voor geprogrammeerde celdood (apoptose) en mede hierdoor is er veel interesse in het proces en de ontregeling van apoptose en tevens in middelen die hierop aan kunnen grijpen. Apoptose kan door verschillende factoren, zoals genomische fouten, stralingsschade of toxische middelen in gang worden gezet. Bovendien zijn er liganden ontdekt die apoptose-inducerende receptoren op cellen kunnen activeren. Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is een van deze liganden en behoort tot de TNF superfamilie. TRAIL initieert apoptose door te binden aan de membraangebonden receptoren DR4 en DR5. De recombinante humane (rh) vorm van TRAIL kan apoptose induceren in veel verschillende soorten kankercellijnen en het interessante is dat het normale cellen niet aantast ⁷. RhTRAIL en afgeleide middelen worden op dit moment in patiënten met uitbehandelde, gemetastaseerde kanker getest en de eerste resultaten tonen dat blootstelling eraan bij mensen gepaard gaat met acceptabele bijwerkingen.

De doelen van het eerste deel van dit proefschrift waren om de effectiviteit van TRAIL in darmpoliepen te onderzoeken, om de regulatie van de TRAIL-receptoren in darmtumoren te bestuderen en om het effect van TRAIL in combinatie met aspirine te bekijken. Het doel van het tweede deel van dit proefschrift was om de veranderingen in proliferatie en apoptose, die voorkomen bij de overgang van goedaardig naar kwaadaardig weefsel in maagpoliepen te bestuderen.

In **hoofdstuk 1** volgt, na een korte inleiding, een beschrijving van de verschillende studies die opgenomen zijn in dit proefschrift. **Hoofdstuk 2** beschrijft de rationale om niet-steroïdale ontstekingsremmers (NSAIDs) te combineren met middelen die specifiek aangrijpen op signaaltransductieroutes in de cel en om deze combinaties te gebruiken in de chemopreventie van darmkanker. Er is herhaaldelijk, in retrospectieve en gerandomiseerde studies aangetoond dat NSAIDs de kans op het ontstaan van poliepen en darmkanker verlagen. Deze middelen geven echter geen volledige bescherming en voor de meeste patiëntengroepen wegen de voordelen niet op tegen de kans op bijwerkingen door NSAID gebruik. Door NSAIDs in lage dosering te combineren met middelen die de effectiviteit kunnen verhogen zouden mogelijk meer patiënten in aanmerking kunnen komen voor chemopreventieve behandeling. In dit hoofdstuk worden de werkingsmechanismen en effectiviteit tegen darmkanker van vier verschillende middelen beschreven alsmede de toegevoegde waarde van de combinatie met NSAIDs. De peroxisome proliferator-activated receptor γ (PPAR γ) liganden, frequent gebruikt in de behandeling van diabetes mellitus, worden als eerste besproken. PPAR γ liganden verminderen de groeisnelheid van darmkankercellijnen. Proefdiermodellen hebben echter wisselende resultaten laten zien ten aanzien van het voorkomen van poliepen, waarbij in sommige modellen behandeling zelfs tot meer poliepen leidde. Deze resultaten moeten eerst verklaard worden voordat klinisch onderzoek kan worden overwogen. Remmers van de cholesterolsynthese, de statines, worden veelvuldig voorgeschreven aan patiënten met een verhoogd cholesterol om de kans op hart- en vaatziekten te verlagen. Net als PPAR γ liganden veroorzaken statines weinig bijwerkingen. Voor statines is aangetoond dat ze de groei van darmkankercellen verminderen en in proefdiermodellen en retrospectieve studies lijken deze middelen een chemopreventief effect te hebben. Middelen die de epithelial growth factor (EGF) signaaltransductieroute inhiberen zijn niet alleen actief tegen darmkanker in cellijnmodellen, maar ook in preklinische en klinische studies. Recent is het eerste monoklonaal antilichaam tegen de EGF-receptor,

cetuximab, geregistreerd voor gebruik in irinotecan resistente darmkanker. RhTRAIL induceert apoptose in humane darmkankercellen, maar niet in normale cellen. Het is ook in het proefdiermodel actief tegen humane tumoren en de eerste vroeg-klinische trials lopen. Hoewel EGF-receptor-remmers en rhTRAIL, net als statines en PPAR γ liganden, effect hebben op darmkankercellen is er voor deze nieuwe middelen nog een lange weg te gaan voordat er voldoende klinische ervaring is, om klinische studies in de preventieve setting te overwegen. Er is voor al deze middelen in cellijn- en/of proefdiermodellen aangetoond dat de activiteit verhoogd wordt indien rhTRAIL gecombineerd wordt met NSAIDs. Concluderend, lijken combinaties van deze verschillende middelen een aantrekkelijke strategie te zijn voor de chemopreventie van darmkanker.

Om de combinatie van NSAIDs en rhTRAIL verder te onderzoeken in een preventieve setting, moet er eerst gekeken worden of naast darmkankercellen, ook adenomen gevoelig zijn voor rhTRAIL-geïnduceerde apoptose. Eerdere studies hebben aangetoond dat de TRAIL receptoren DR4 en DR5 hoger tot expressie komen in adenomen dan in normaal darmepitheel. **Hoofdstuk 3** beschrijft onderzoek naar de gevoeligheid van humane adenoomcellijnen en humane adenomen voor rhTRAIL-geïnduceerde apoptose. Twee adenoomcellijnen werden blootgesteld aan rhTRAIL. Dit resulteerde in tot 55% apoptose en deze apoptose ging samen met caspase-8 en caspase-3 activatie. De apoptose kon geremd worden door een breedspectrum caspase-remmer, wat aangeeft dat deze apoptose caspase-afhankelijk is. Omdat er veel discussie bestaat over de validiteit van adenoomcellijnen als model voor humane adenomen, is er ook gekeken naar het effect van rhTRAIL in kortetermijnkweken van humane adenomen en bipten van normaal darmepitheel. Behandeling met rhTRAIL leidde tot meer caspase-afhankelijke apoptose in adenomen met hooggradige dysplasie (n = 21) in vergelijking met onbehandelde controles, gemeten met behulp van morfologische criteria en immunohistochemie voor actief caspase-3. In adenomen met laaggradige dysplasie (n = 17) en in bipten van normaal darmepitheel (n = 15) resulteerde behandeling met rhTRAIL niet in meer apoptose. Deze resultaten geven aan dat humane adenoomcellijnen en humane adenomen met hooggradige dysplasie wel gevoelig zijn voor rhTRAIL-geïnduceerde apoptose in tegenstelling tot humane adenomen met laaggradige dysplasie en normaal darmepitheel. Daaruit blijkt dat rhTRAIL mogelijk gebruikt zou kunnen worden voor de behandeling van adenomen met hooggradige dysplasie. Dit is vooral interessant voor patiënten met erfelijke vormen van darmkanker en grote adenomen. Als rhTRAIL-behandeling deze adenomen zou kunnen verkleinen

zodat ze endoscopisch te verwijderen zijn, zou chirurgische verwijdering van de darm vermeden of uitgesteld kunnen worden.

In hoofdstuk 3 hebben we gezien dat de TRAIL-receptoren in adenomen functioneel zijn en blootstelling aan rhTRAIL tot apoptose kan leiden. In de toekomst zal rhTRAIL-geïnduceerde apoptose mogelijk gebruikt worden in de behandeling van darmpoliepen en darmkanker en het is daarom belangrijk om te begrijpen waarom de TRAIL-receptoren in darmpoliepen en darmkanker verhoogd tot expressie komen in vergelijking met normaal darmweefsel. Dit wordt in **hoofdstuk 4** onderzocht. De Wnt/APC/ β -catenine signaal transductieroute is belangrijk bij het ontstaan van darmkanker. Door een mutatie in het *adenomatous polyposis coli* (APC) gen kan het eiwit β -catenine niet goed worden afgebroken. β -catenine hoopt zich dan op in het cytoplasma van de cel, wordt getransloceerd naar de celkern en bindt uiteindelijk aan de transcriptie factor T-cell factor (TCF). Dit resulteert in de activatie van genen die invloed hebben op onder andere de celdeling en apoptose. Het is mogelijk dat ook de TRAIL-receptoren op deze manier beïnvloed worden. Om dit te onderzoeken werd de hoeveelheid β -catenine in carcinoom en adenoomcellen verlaagd, gebruikmakend van RNA-interferentie. Wij laten zien dat verlaging van de hoeveelheid β -catenine in de cel leidt tot minder DR4/5 eiwit in de cel en tot verminderde expressie van deze receptoren op de celmembraan in darmkankercellijnen. Verlaging van de hoeveelheid β -catenine leidde niet alleen tot minder DR4 en DR5, maar ook tot een verhoogde gevoeligheid voor rhTRAIL. Om te bekijken of deze effecten afhankelijk zijn van de TCF transcriptiefactor, werd dominant negatief TCF-4 tot overexpressie gebracht. Deze β -catenine onafhankelijke vermindering van de TCF-4 activiteit had echter geen invloed op de TRAIL-receptor-expressie of rhTRAIL-gevoeligheid. Om dit verder te onderzoeken werd gekeken naar de associatie tussen de eiwitexpressie van β -catenine en DR4/5 in humane adenomen en carcinomen. Afwijkende expressie van β -catenine in de kern en/of het cytoplasma, ging gepaard met een hoger percentage cellen met DR4 en DR5 expressie. In opeenvolgende coupes van adenomen werd aangetoond dat afwijkende β -catenine overexpressie co-localiseerde met verhoogde expressie van DR4 en DR5. In humane colorectalecarcinomen was afwijkende β -catenine in de meerderheid van de tumoren aanwezig en de DR4/5 expressie was met name homogeen. Deze resultaten geven aan dat de geleidelijke verhoging van de expressie van DR4/5 in de colorectale carcinogenese voor een deel veroorzaakt wordt door β -catenine expressie, maar dat dit onafhankelijk is van de TCF-transcriptiefactor. Het is van belang om de regulatie van de TRAIL-receptoren beter te begrijpen aangezien kennis hiervan kan helpen om de

effectiviteit en potentiële geneesmiddeleninteracties van rhTRAIL in combinatie met andere middelen te voorspellen.

In hoofdstuk 2 werd beschreven dat NSAIDs en rhTRAIL mogelijk een synergistische werking hebben. In **hoofdstuk 5** bekeken we of de NSAID aspirine de effectiviteit van rhTRAIL in darmkankercellijnen kan vergroten. Ondanks het feit dat veel darmkankercellijnen gevoelig zijn voor rhTRAIL-geïnduceerde apoptose worden ook intrinsieke en verworven resistentie beschreven. Wij bekeken verschillende humane darmkankercellijnen met wisselende gevoeligheid voor rhTRAIL, een cellijn die rhTRAIL-resistent is gemaakt en een cellijn waarin dominant negatief TCF-4 tot expressie kan worden gebracht. Voorbehandeling met aspirine (tot 10 mM) veroorzaakte een concentratie-afhankelijke verhoging in de gevoeligheid (factor 2-9) in alle cellijnen voor rhTRAIL-geïnduceerde apoptose. Ook de resistente cellijnen konden op deze manier weer gevoelig worden gemaakt voor rhTRAIL. Deze apoptose ging samen met verhoogde expressie van M30, een product van caspase-activiteit in epitheliale cellen, en verhoogde activatie van caspase-3. Overexpressie van dominant negatief TCF-4 blokkeerde geheel het sensitiserende effect van aspirine op rhTRAIL-geïnduceerde apoptose compleet. Deze resultaten geven aan dat darmkankercellijnen met intrinsieke of verworven resistentie voor rhTRAIL gevoelig gemaakt kunnen worden voor rhTRAIL-geïnduceerde apoptose met behulp van aspirine en dat dit effect deels via de TCF-4 transcriptiefactor verloopt.

Voor de ontwikkeling van geneesmiddelen die ingrijpen op specifieke signaaltransductieroutes in (pre)maligne tumoren is het belangrijk om te begrijpen wat voor routes een rol spelen in de verandering van goedaardige naar kwaadaardige cellen. Een manier om dit aan te pakken is door te bestuderen waarom bepaalde veranderingen in sommige gevallen leiden tot dysplasie en maligniteit en in andere gevallen niet. De laatste drie hoofdstukken beschrijven studies naar fundic gland poliepen (FGPs). FGPs zijn de meest voorkomende maagpoliepen en worden in ongeveer 2% van de bevolking gevonden. Ze worden voornamelijk gevonden in het corpus en de fundus van de maag. Er werd lange tijd gedacht dat deze poliepen volkomen goedaardig waren ⁸. In patiënten met familiale adenomateuze polyposis (FAP), een erfelijke vorm van darmkanker, komen deze maagpoliepen veel vaker voor en kan zich er hooggradige dysplasie in ontwikkelen, met soms zelfs ontaarding in kanker. In **hoofdstuk 6** beschrijven we een 68 jarige patiënt met sporadische, niet-FAP gerelateerde, FGPs waarin hooggradige dysplasie werd gevonden. Hooggradige dysplasie in een FGP werd nooit eerder

beschreven in een patiënt zonder FAP. Twee colonoscopieën hadden bij deze patiënt de diagnose FAP uitgesloten. Aanvullende immunohistochemische kleuringen toonden afwijkende β -catenine expressie, verhoogde proliferatie en verhoogde apoptose in de dysplastische gebieden suggererend dat de Wnt pathway hier actief was. Deze casus geeft aan dat maligne ontaarding in FGPs niet voorbehouden is aan FAP-geassocieerde FGPs.

De verschillen in kans op maligne ontaarding tussen FGPs van patiënten met en zonder FAP werd verder bestudeerd in **hoofdstuk 7**. FAP-geassocieerde FGPs ontstaan, net als de colorectale adenomen en carcinomen bij deze patiënten, door een mutatie in het *APC* gen. FGPs bij andere mensen ontstaan door mutaties in het *β -catenine* gen. Dysplasie in FGPs van patiënten zonder FAP, wat minder vaak voorkomt dan in FAP-geassocieerde FGPs, wordt vaak geassocieerd met *APC* in plaats van *β -catenine* mutaties. Deze data suggereren dat *APC* en *β -catenine* mutaties verschillende functionele consequenties hebben. Om deze hypothese verder te onderzoeken hebben we met behulp van immunohistochemie gekeken naar proliferatie, apoptose, β -catenine expressie en expressie van p53, een eiwit nauw betrokken bij onder andere celcyclus controle en apoptose, in FGPs van patiënten met en zonder FAP. Als er geen dysplasie aanwezig was hadden beide types FGP vergelijkbare proliferatieve activiteit, mate van apoptose, β -catenine expressie en p53 expressie. Zoals verwacht, werd dysplasie vaker gezien in FAP-geassocieerde FGPs (4/9) dan in FGPs van patiënten zonder FAP (1/18). In FGPs met dysplasie was er afwijkende β -catenine expressie in 3/5 gevallen. Verder was er een verhoogde proliferatieve activiteit en een hogere mate van apoptose dan in de niet-dysplastische gebieden. Overexpressie van p53 werd niet gezien. De vergelijkbare proliferatieve activiteit en mate van apoptose in beide typen FGPs ondergraaft de hypothese dat *APC* and *β -catenine* mutaties verschillende functionele consequenties hebben in FGPs. De associatie tussen gebieden met dysplasie en hoge proliferatie en apoptose samen met kernkleuring voor β -catenine geeft aan dat er sprake is van activatie van de Wnt-APC- β -catenine pathway in gebieden met dysplasie. Het feit dat niet in alle FAP-geassocieerde FGPs dysplasie werd gevonden geeft aan dat er, naast *APC* mutaties, andere factoren een rol spelen in de ontwikkeling van dysplasie in FGPs.

FGPs worden meestal per toeval ontdekt tijdens endoscopie, aangezien ze geen maagdarmlachten veroorzaken. De aanwezigheid van dysplasie in een deel van de FGPs heeft tot vragen geleid met betrekking tot de klinische consequenties van deze poliepen. Deze discussie is met name relevant in

patiënten die protonpompremmers (PPIs) gebruiken om de zuurexcretie in de maag te verminderen. Vanaf de introductie van PPIs in de jaren tachtig zijn er meldingen geweest van een mogelijk verhoogd risico op de ontwikkeling van FGPs tijdens langdurig PPI-gebruik. Twee grote studies die dit onderzochten hebben tot tegenstrijdige resultaten geleid. **Hoofdstuk 8** beschrijft een case-control studie naar de prevalentie en lengte van PPI gebruik alsmede de prevalentie van FGPs in opeenvolgende patiënten die een gastroduodenoscopie ondergingen. Er werden data van 599 patiënten verzameld, waarvan 322 PPIs gebruikten en 107 histologisch bevestigde FGPs hadden. Langdurig gebruik van PPIs was geassocieerd met een verhoogd risico op FGPs (1 – 4,9 jaar: Odds Ratio (OR) 2,2, 95% betrouwbaarheidsinterval (BI) 1,3 – 3,8 en 5 jaar of langer: OR 3,8, 95% BI 2,2 – 6,7) maar kortere behandeling met PPIs (< 1 jaar) niet (OR: 1,0, 95% BI 0,5 – 1,8). Biopsieën van de FGPs werden bestudeerd en laaggradige dysplasie werd in 1/107 FGPs aangetoond. FGPs van patiënten met langdurige PPI gebruik hadden een relatief groter cysteus oppervlak en dit ging samen met parietaalcelhyperplasie en uitstulpingen van de parietaalcellen. Dit suggereert dat de FGPs waarschijnlijk ontstaan als gevolg van de zuurremmende therapie. Concluderend, de prevalentie van FGPs lijkt hoger te zijn in patiënten met langdurige behandeling met PPIs. Echter de kans op dysplasie is verwaarloosbaar en het lijkt daarom niet nodig om deze patiënten langdurig endoscopisch te vervolgen.

Conclusies en Toekomstperspectieven

Specifieke apoptose-inductie is potentieel aantrekkelijk als onderdeel van de behandeling van het colorectaalcarcinoom aangezien deze aandoening gekenmerkt wordt door een relatieve resistentie voor apoptose en een beperkte respons op traditionele chemotherapie. De cytokine rhTRAIL kan apoptose induceren in colorectale adenoom- en carcinoomcellijnen evenals in kortetermijnkweken van adenomen.

RhTRAIL-gebaseerde therapieën maken gebruik van het verschil in TRAIL-gevoeligheid tussen normale en tumorcellen, dat deels gebaseerd is op verschillende expressie van de TRAIL-receptoren. Colorectale adenomen en carcinomen brengen de pro-apoptotische TRAIL-receptoren DR4 en DR5 hoger tot expressie dan normaal colonepitheel. Colorectale tumoren brengen dus receptoren tot expressie die hen blootstelt aan de inductie van

apoptose. Wij hebben aangetoond dat overexpressie van β -catenine, dat een belangrijke rol speelt in de colorectale carcinogenese, ook een rol speelt in de verhoging van de expressie van de TRAIL-receptoren. Begrip van receptorregulatie is niet alleen belangrijk om te bepalen welke combinaties van middelen de receptorexpressie of receptorfunctionaliteit optimaliseren, maar ook om te voorspellen van welke combinaties mogelijk additionele bijwerkingen te verwachten zijn. Zo werd bij vrouwen met borstkanker gezien dat het gebruik van trastuzumab, een monoklonaal antilichaam gericht tegen de EGF-receptor HER-2, met name in combinatie met anthracycline-gebaseerde chemotherapie, bij een deel van de vrouwen leidde tot cardiotoxiciteit, mogelijk door opregulatie van de HER-2 receptor in het myocard⁹. Huidig onderzoek dat zich richt op de mechanismen van TRAIL-receptorregulatie, de rollen van de verschillende receptoren in TRAIL-geïnduceerde apoptose en de affiniteiten van de verschillende receptoren voor TRAIL en zijn afgeleiden, is daarom erg belangrijk^{10,11}.

Niet alle coloncarcinoomcellijnen zijn gevoelig voor rhTRAIL en in ons ex-vivo model waren alleen adenomen met hooggradige dysplasie gevoelig voor rhTRAIL. Het is belangrijk om de redenen voor deze verschillen in rhTRAIL-gevoeligheid te begrijpen. Niet alleen om de activiteit van rhTRAIL in verschillende situaties te kunnen voorspellen, maar ook om manieren te vinden om de rhTRAIL-sensitiviteit te verhogen. rhTRAIL-gevoeligheid kan beïnvloed worden door verschillende factoren, inclusief *p53* mutatie-status, *p21*, fosfatase en tensin homolog (PTEN) expressie en ook door de aanwezigheid van de componenten van de TRAIL-apoptose route¹¹⁻¹³. Huidig onderzoek wijst op een belangrijke rol voor de ratio tussen caspase-8 en cellulair FLICE-like inhibitory protein (c-FLIP) in het bepalen van de gevoeligheid voor rhTRAIL. Ook het co-localiseren van TRAIL-receptoren in lipid-rafts in de celmembraan en het verlagen van expressie van anti-apoptotische eiwitten zoals Bcl-2 en X-linked inhibitor of apoptosis protein (XIAP) lijken belangrijk. *In vitro* kan de rhTRAIL-gevoeligheid niet alleen door aspirine worden verhoogd, maar ook met verschillende traditionele chemotherapeutica en nieuwe middelen zoals proteasoomremmers¹². Middelen die de gevoeligheid voor rhTRAIL verhogen grijpen meestal aan op een of meerdere van de bovengenoemde factoren, echter de exacte mechanismen zijn meestal nog onduidelijk¹². Het optimaliseren van kortetermijnkweek systemen is nuttig omdat de gevoeligheid voor apoptose-inductie door verschillende (combinaties van) middelen en ook de daarbij betrokken signaaltransductieroutes bestudeerd kunnen worden in vers patiëntenmateriaal. Toekomstige studies naar de effecten van rhTRAIL, als monotherapie en in combinatie met andere middelen, in proefdiermodellen

van sporadische en erfelijke darmkanker kunnen tevens de basis leggen voor het gebruik van TRAIL-gebaseerde therapieën in colorectaalcarcinoom.

Er lopen op dit moment fase I/II studies met TRAIL-gebaseerde therapieën zoals rhTRAIL en ook agonistische antilichamen tegen de pro-apoptotische TRAIL receptoren in kankerpatiënten ¹⁴. Als de resultaten betreffende de gunstige toxiciteitsprofielen voor TRAIL-gebaseerde therapie blijven bestaan, zouden ook fase II studies om de effecten van rhTRAIL op adenomen in FAP-patiënten te onderzoeken mogelijk interessant worden. Kortetermijnkweken van biopten van colorectaleadenomen of -carcinomen kunnen mogelijk helpen om rhTRAIL-gevoeligheid bij de individuele patiënt te voorspellen.

Voor klinische studies met rhTRAIL en andere nieuwe, gerichte antitumor middelen, wordt het waarschijnlijk steeds belangrijker om per patiënt de juiste behandeling te kunnen selecteren op grond van eigenschappen van de tumor. Bij patiënten met borstkanker worden therapie keuzes gemaakt op grond van de oestrogeen- en progesteronreceptoren, evenals de HER-2 receptor ¹⁵. Hoewel er een aantal prognostische factoren in colorectaalcarcinoom zijn geïdentificeerd, zijn er nog geen moleculaire determinanten van de effectiviteit van chemotherapeutische strategieën ontdekt ¹⁶. Dit geeft aan dat een complexe combinatie van factoren waarschijnlijk de gevoeligheid bepaalt. Om deze reden is het essentieel om de mechanismen van de carcinogenese, evenals de mechanismen die bepalend zijn voor effectiviteit of resistentie van geneesmiddelen in colorectaalcarcinoom te blijven bestuderen. Dit zou kunnen leiden tot het bepalen van moleculaire karakteristieken van de tumor die dan kunnen helpen bij het kiezen van de optimale behandelingsstrategie.

Combinaties van traditionele chemotherapeutica en middelen die specifiek ingrijpen op verstoorde signaaltransductieroutes in kankercellen blijken een interessante strategie als antitumor behandeling bij het coloncarcinoom. Na vele jaren van onderzoek naar signaaltransductie in kankercellen, zijn recent de eerste twee gerichte therapieën, monoklonale antilichamen respectievelijk gericht tegen de EGF-receptor-1 en vascular endothelial growth factor, geregistreerd voor de behandeling van gemetastaseerd colorectaalcarcinoom ¹⁷. In de verdere ontwikkeling van combinaties van (gerichte) antitumor middelen, zoals rhTRAIL, is kennis van de moleculaire mechanismen achter carcinogenese, geneesmiddeleffectiviteit en geneesmiddeleninteracties essentieel.

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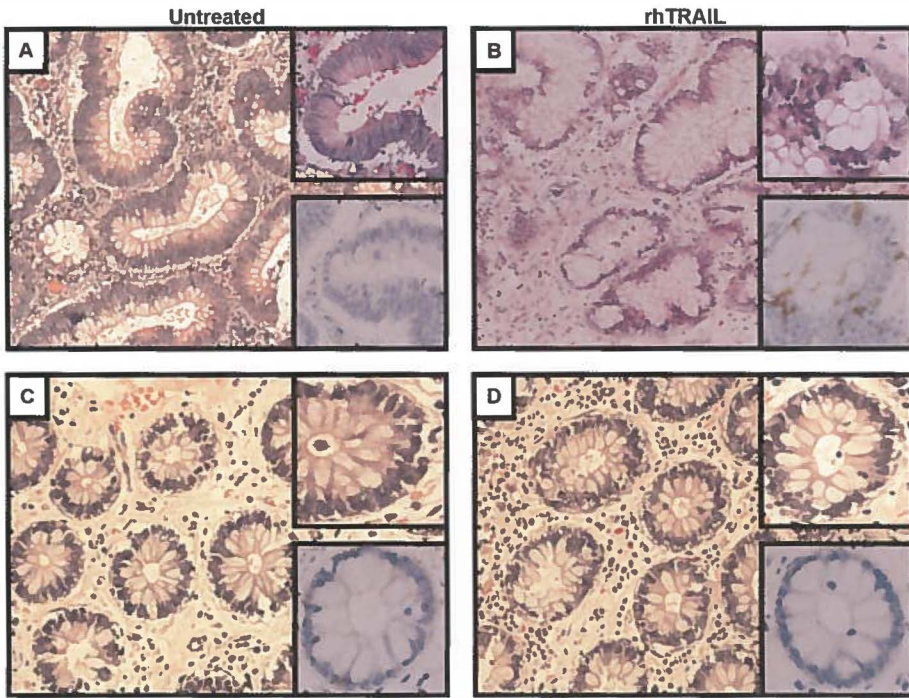
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Appendix 1

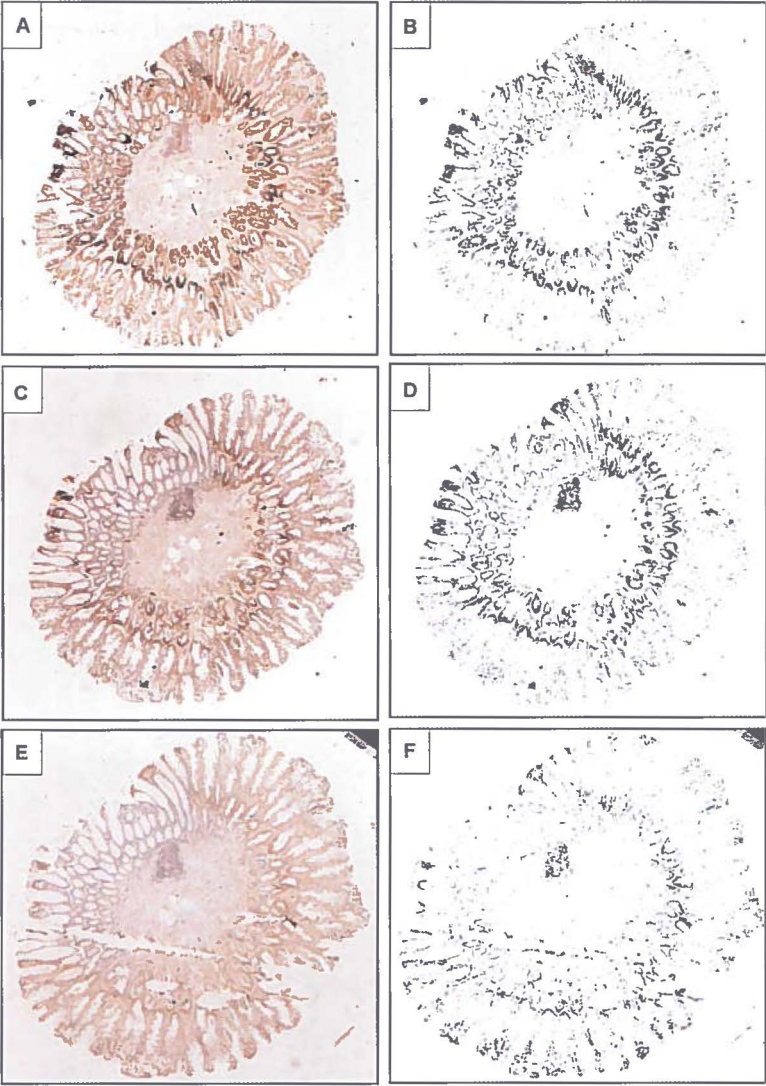
Colour figures for chapter 3



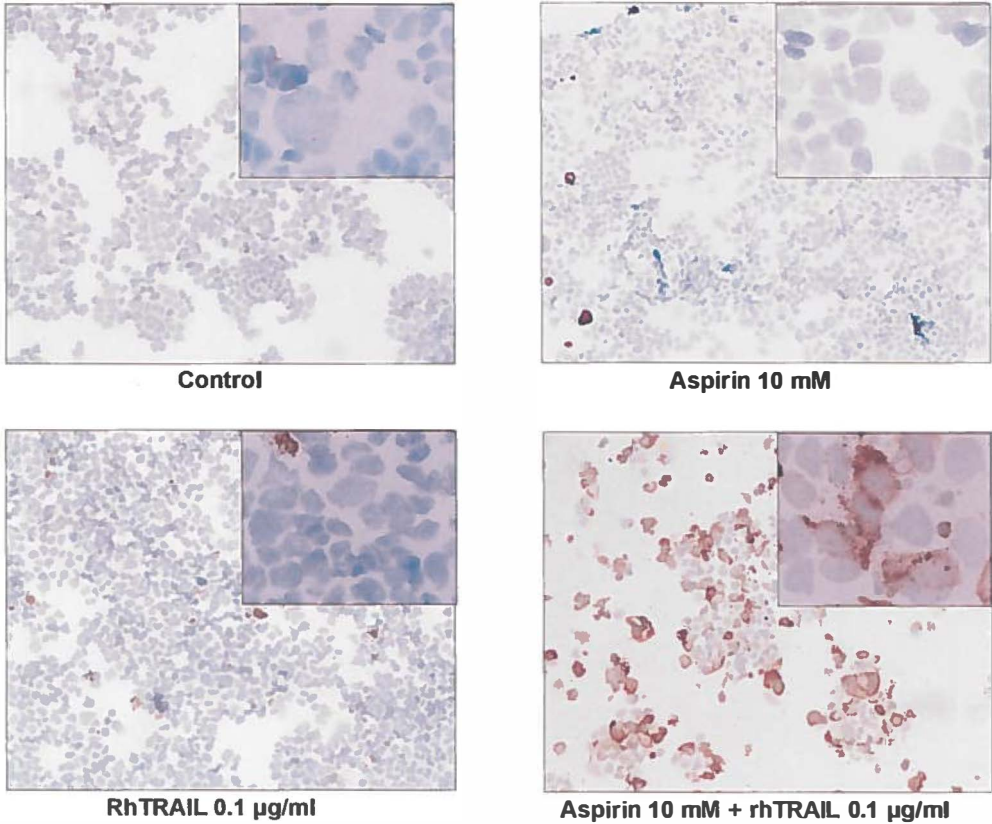
Chapter 3, figure 2: Colon tissue sections after 5 hours of incubation. Main figures: H&E stained (original magnification 200x). Up per inserts: H&E stained (original magnification 400X). Lower inserts: stained for cleaved caspase 3 (original magnification 400X). Adenoma with high-grade dysplasia: (A) untreated and (B) treated with 1 $\mu\text{g/ml}$ rhTRAIL. Normal colon epithelium: (C) untreated and (D) treated with 1 $\mu\text{g/ml}$ rhTRAIL..

Appendix 2

Colour figures for chapters 4 and 5



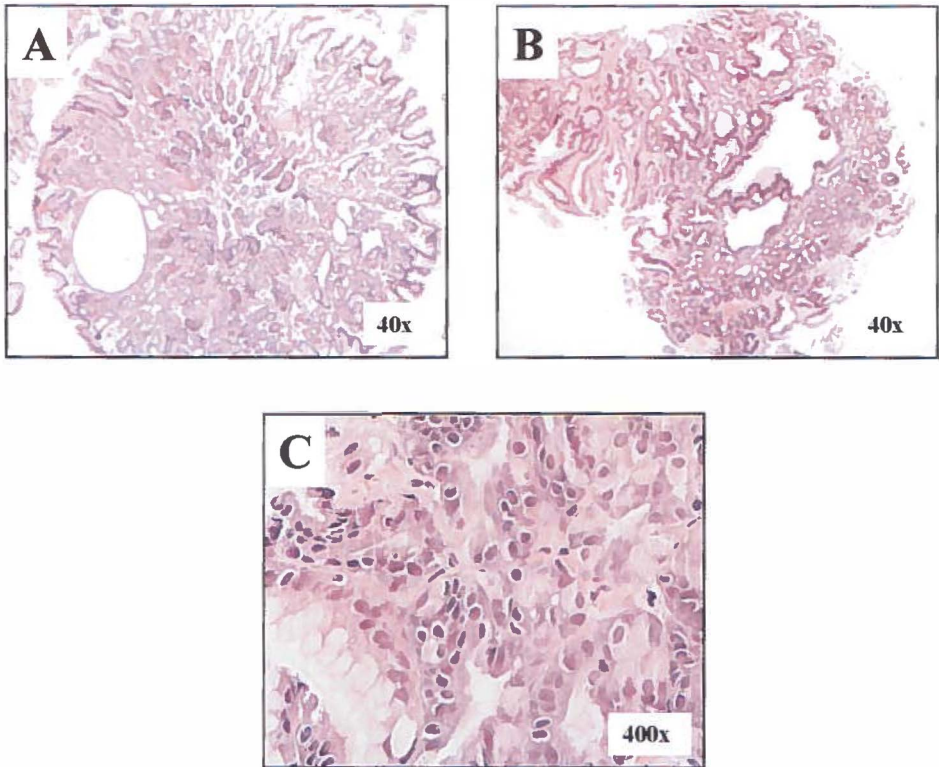
Chapter 4, figure 4: β -catenin (A, B), DR4 (C, D) and DR5 (E, F) stained tissue sections of an adenoma showing co-localization of staining, before (A, C, E) and after (B, D, F) accentuation of contrast. Original magnification 25x.



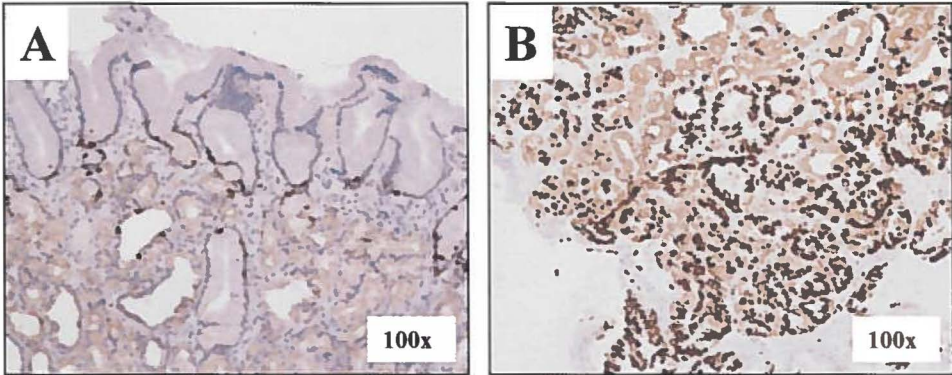
Chapter 5, figure 2C: Apoptosis in the CACO-2 cell line after incubation with aspirin and/or rhTRAIL measured using M30 staining. M30 positive cells stain brown. Original magnification main figures: 40x. Original magnification inserts: 400x. One representative of at least three independent experiments is shown.

Appendix 3

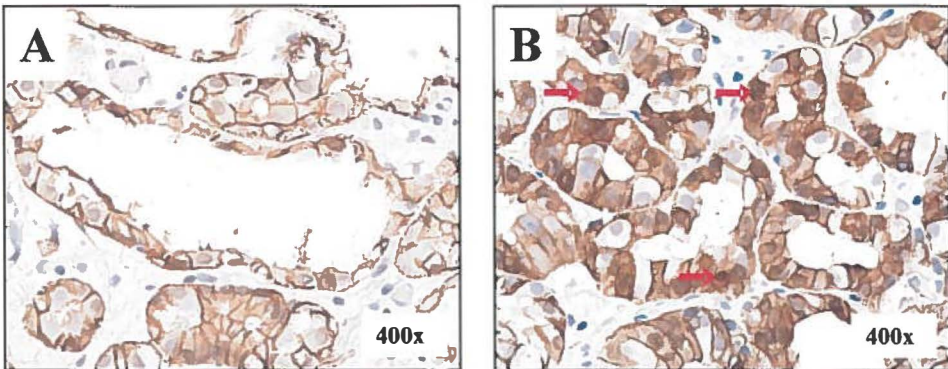
Colour figures for chapter 6



Chapter 6, figure 1: (A) Histopathological appearance of one of the fundic gland polyps without dysplasia (H&E, magnification x40). (B) Histopathological appearance of one of the fundic gland polyps containing high-grade dysplasia characterized by nuclear hyperchromatism and stratification and irregular size (H&E, magnification x40). (C) Detail of dysplastic area of FGP shown in B (H&E, magnification x400).



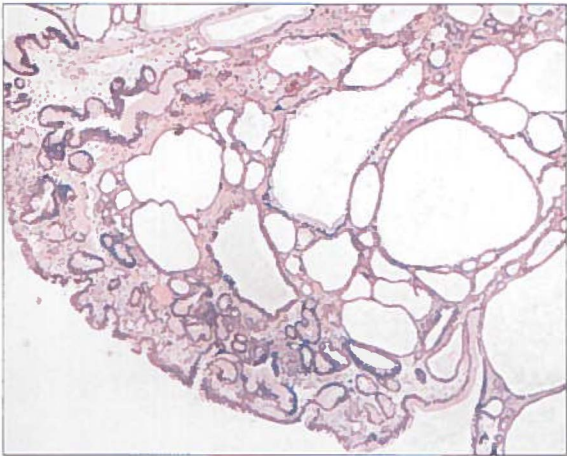
Chapter 6, figure 2: Immunostaining for proliferation with Ki-67. (A) One of the FGPs without dysplasia, proliferation is confined to the neck stem cell region (magnification x100). (B) One of the FGPs with high-grade dysplasia, the proliferative area extends beyond the neck stem cell region (magnification x100).



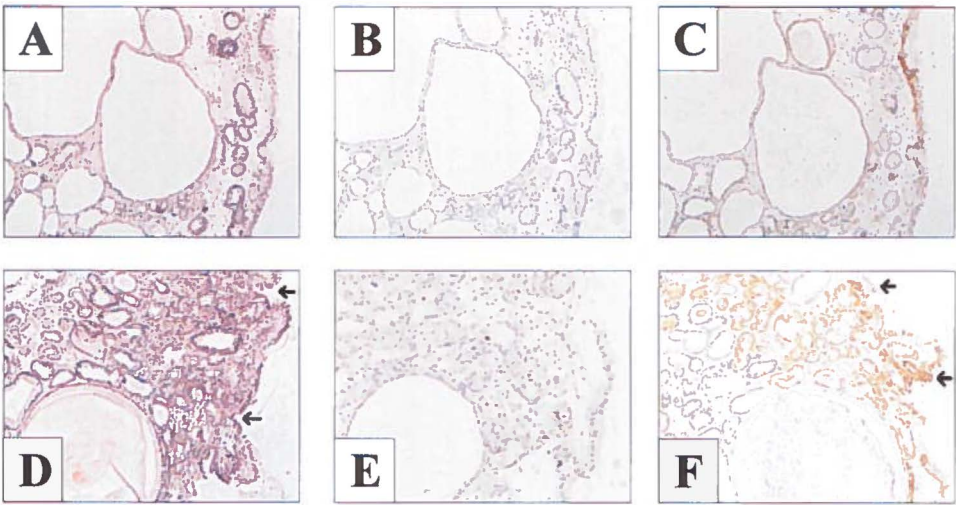
Chapter 6, figure 3: Immunostaining for β -catenin in the FGPs. (A) Fundic gland polyp without dysplasia showing mainly membranous and some cytoplasmic staining (magnification x400). (B) Fundic gland polyp containing high-grade dysplasia and nuclear β -catenin staining, with arrows showing examples of positively stained nuclei (magnification x400).

Appendix 4

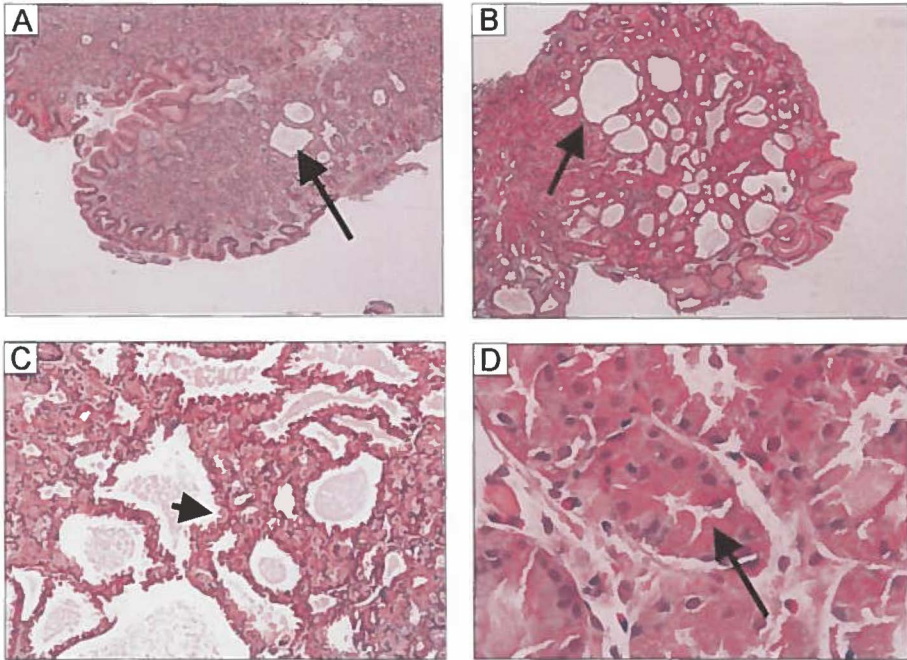
Colour figures for chapters 7 and 8



Chapter 7, figure 1. Typical histopathological appearance of a fundic gland polyp, showing multiple dilated cysts.



Chapter 7, figure 2. Example of a fundic gland polyp without dysplasia (A-C) and a fundic gland polyp with low-grade dysplasia (D-F). Serial hematoxylin-eosin (HE) staining (A, D) and immunohistochemical staining of MIB-1 (B, E) and β -catenin (C, F). In the FGP without dysplasia, relatively low MIB-1 positivity and membranous β -catenin staining is seen whereas a FGP with dysplasia (arrows) shows high proliferative activity and nuclear β -catenin staining.



Chapter 8, figure 2: H&E stained tissue sections of fundic gland polyps (FGPs). (A) FGP from a patient who had never used proton pump inhibitors (PPIs) showing a few small cysts (arrow), original magnification 40x. (B) FGP from a patient with more than 1 year of PPI use showing a large number of cysts (arrow), original magnification 40x. (C) FGP from a patient with more than 1 year of PPI use showing parietal cell hyperplasia and parietal cell protrusions (arrow), original magnification 100x. (D) FGP from a patient with more than 1 year of PPI use showing parietal cell hyperplasia and parietal cell protrusions (arrow), original magnification 400x.

